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CHARACTERIZATION BY NMR AND FLUORESCENCE SPECTROSCOPY OF DIFFERENCES IN THE CONFORMATION OF NON-AGED AND AGED ORGANOPHOSPHORYL CONJUGATES OF AChE

FINAL REPORT

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The objective of this study was to detect and characterize by nuclear magnetic resonance (nmr) and optical spectroscopy and by X-ray crystallographic study structural differences between non-aged and aged organophosphotyl (OP) conjugates of acetylcholinesterase (AChE) and chymotrypsin (Cht) which might help to explain the unexpected resistance of the aged form to commonly used reactivators. During the period covered by this report we synthesized novel organophosphate inhibitors of serine hydrolases, performed spectroscopic studies on the conformational stabilization of aged and non-aged OP-Cht and OP-AChE conjugates and investigated OP-Cht conjugates by ^{31}P-nmr spectroscopy. In addition, we prepared crystalline OP-Cht conjugates and determined their structure by X-ray crystallography. The various experimental

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techniques employed all point to the formation of a salt-bridge between the negatively charged oxygen atom produced by aging and imidazolium group within the catalytic site of Cht (and presumably also of cholinesterases). This clearly explains the increased conformational stability of the aged enzyme. It does not, however, offer an explanation for the unusual resistance to nucleophilic reactivation. Results provided evidence that dealkylation with concomitant production of a P-O bond is not a prerequisite for 'aging'. Displacement reactions at phosphorus atoms are believed to proceed by a pentacoordinate intermediate. It is possible that the salt-bridge produced by aging does not permit the substituents around the P atom to assume the required geometry for displacement to occur.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

This work was carried out with the collaboration of J. Grunwald, M. Harel, E. Roth, N. Steinberg, C.-T. Su, J.L. Sussman, and D. Waysbrot.

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OBJECTIVES

1. General

The objective of this project was to characterize, by nmr and fluorescence spectroscopy and by X-ray crystallography, conformational differences between aged and non-aged organophosphoryl conjugates of acetylcholinesterase (AChE) and chymotrypsin (Cht). Such differences may help to explain the unexpected resistance of the aged forms to commonly employed reactivators.

2. Specific Aims

In our Midterm Report (January 1, 1988), which covered the period September 1987-August 1988, we focused our efforts on the following principal lines of research:

a. Preparation of stoichiometric aged and non-aged organophosphoryl conjugates of Cht and comparison of their conformational stability.

b. Synthesis of novel 2-naphthol-containing fluorescent organophosphates.

c. Preliminary studies of the inhibition of Torpedo AChE by pyrene-containing fluorescent organophosphates.

d. Preparation and X-ray studies of organophosphoryl conjugates of Cht in the crystalline state.

The present report covers the second period, September 1988- November 1989. During this period, which included a 3-month extension, we were engaged in the following principal lines of research:

a. Synthesis of novel 2-naphthol-containing fluorescent organophosphates.

b. Synthesis of organophosphate inhibitors of Cht and their utilization for large-scale preparation of OP-Cht conjugates.

c. Preparation of pyrene-containing OP-AChE conjugates.

d. Spectroscopic studies on the conformational stabilization of aged and non-aged OP-Cht and OP-AChE conjugates.

e. ^{31}P-nmr spectroscopy studies of OP-Cht conjugates.

f. Preparation of crystalline OP-Cht conjugates and determination of their structure by X-ray crystallography.

This report presents findings obtained after the midterm of the grant, and summarizes the entire grant effort.

BACKGROUND

Many serine hydrolases, such as acetylcholinesterase (AChE), chymotrypsin (Cht) and trypsin, are inhibited irreversibly by organophosphorus (OP) esters. Inhibition is achieved by formation of a stoichiometric (1:1) covalent conjugate with the active site serine (1).

The powerful acute toxicity of these compounds is attributed primarily to their irreversible inhibition of AChE (2). Although poisoning by certain OP's can be treated effectively by various oxime reactivators which detach the phosphoryl moiety from the serine hydroxyl of the enzyme (3, 4), for other OP's, reactivation may be hampered by a parallel competing reaction which transforms the inhibited AChE into an "aged" form which can no longer be regenerated by the commonly used reactivators (5-7). This aging process is particularly pronounced for OP-AChE conjugates in which the OP moiety contains a secondary alkyl group (1), as is the case for conjugates produced by use of diisopropylphosphorofluoridate (DFP), isopropyl methylphosphonofluoridate (sarin) and pinacolyl methylphosphonofluoridate (soman). The inability to regenerate aged organophosphoryl-AChE conjugates with such oximes renders therapy of intoxication by such OP's notoriously difficult (8, 9). Although aging may occur by either acid (10, 11) or base catalysis (12, 13), it is usually accepted that the common denominator is the loss of an alkyl group from the bound OP moiety, as depicted in Scheme 1.

$$EH + XP(O)(OR')R \xrightarrow{-HX} E\text{-}P(O)(OR')R \xrightarrow{-R'} E\text{-}P(O)(O^-)R$$

where: R= alkyl, aryl, alkyloxy or aryloxy

R'= alkyl, aryl

X= F, Cl, p-nitrophenoxy, dialkylaminoethanthiol

SCHEME 1

It has been suggested that the negative charge resulting from the aging reaction imposes an electrostatic barrier to nucleophilic attack on the phosphorus atom by the oximate anion of the reactivator (14). However, kinetic studies with model phosphate ester analogs of the assumed aged and non-aged conjugates indicate that the negative charge would retard reactivation by no more than 50 to 100-fold (15), whereas, in fact, reactivation of aged enzyme is not experimentally detectable (7, 11).

We postulated (16) that the observed resistance to reactivation might result from a conformational change in the enzyme occurring concomitantly

with aging, and that such a conformational change might be sought by use of suitably designed organophosphate probes in combination with an appropriate physicochemical technique. Fluorescent probes are particularly suitable for detecting changes in the microenvironment of the moiety to which they are attached (e.g., hydrophobicity, microviscosity, chirality and local pH). We have thus been able to provide experimental evidence for the conformational changes postulated, both for AChE and Cht, by use of suitable fluorescent OP's designed and synthesized for this purpose, (16, 17). In parallel, for Cht, we were able to establish, by use of ^{31}P-NMR spectroscopy, that the phosphate moiety in the conjugates under study was in the form of a triester and a diester in the non-aged and aged conjugates, respectively, as predicted (18). It was hoped thus that we could clarify and illuminate the structural relationship between the phosphoryl residue and the protein backbone of aged serine esterases relative to their non-aged counterparts.

Although our eventual interest is in understanding the active-site geometry of the aged OP-AChE conjugate relative to its non-aged counterpart, as already mentioned, part of this study was carried out on Cht, which, like AChE and other serine hydrolases, is readily inhibited by DFP and other OP's. We felt that studies of this type would provide very useful information since Cht is a well-characterized enzyme whose sequence and three-dimensional structure have been fully elucidated (19). Furthermore, Cht is commercially available in highly purified form in large quantities. This renders various experimental approaches readily accessible, thus allowing information concerning the aging process to be directly obtained for OP-Cht conjugates, as well as allowing us to evaluate the feasibility of applying the same approaches to AChE itself.

Most of our fluorescence spectroscopy studies to date have utilized pyrene-containing OP conjugates of AChE (16) and Cht (17). Although much valuable information has been obtained by use of this fluorophore, it suffers from various drawbacks; in particular, it is relatively insensitive to its environment with respect to both its absorption and emission wavelengths. In this project we describe the synthesis and initial characterization of a new class of fluorescent OP's in which the fluorophore is 2-naphthol (20). This probe displays an excited state ionization constant very different from that in the ground state, and is known to be highly conformation-sensitive when bound to proteins. It is, therefore, a promising candidate for detecting aging-associated conformational changes, as will be discussed below.

In this report we will also present data obtained by an experimental approach which provides direct evidence that aging is accompanied by an enhanced stabilization of the OP-enzyme conjugate, in line with data presented earlier for serum cholinesterase by Masson and Goasdue (21). For this purpose we initially adopted the same technique employed by these authors, namely the transverse urea gradient electrophoresis technique of Creighton (22). Subsequently we monitored protein unfolding more directly by measuring changes in the circular dichroism (CD) spectrum of the aged and non-aged OP conjugates as a function of increasing guanidine hydrochloride concentration. We have obtained evidence, using both techniques, to show that aged conjugates of Cht and AChE are substantially more resistant to denaturation than homologous non-aged conjugates.

^{31}P-nmr was used in the past by ourselves (18) and others (23, 24) to

provide direct evidence for the presence of a P-O^- bond in aged conjugates of Cht. In the past year we extended the use of this technique to investigate whether aging via dealkylation is accompanied by alkylation of neighboring amino-acid side-chains within the OP-Cht conjugate, a possibility which was raised in our original proposal. The technique of nmr spectroscopy has also been utilized in the past decade to study changes in the pK_a of the catalytic histidine in a number of serine hydrolases. Various adducts of active-site-directed reagents were studied by ^1H-, ^{13}C-, and ^{19}F-nmr, and evidence was accumulated that adduct formation involved strong interaction of the $N^{\varepsilon 2}$ of the histidine with putative transition-state analog structures. This was reflected in an apparent increase in the pK_a of this histidine - His57 in the case of Cht (for further details and references see Results and Discussion). We decided, therefore, to expand our ^{31}P-nmr measurements to the alkaline range in an endeavor to obtain a chemical shift pH-dependence for the aged OP-Cht conjugates which could be compared with the corresponding data for the non-aged conjugates. This enabled us to consider an additional explanation for the resistance both to unfolding and to reactivation of the aged OP conjugates.

X-ray crystallography is the only experimental approach currently available which can yield direct information concerning the three-dimensional structure of a protein and for thereby detecting conformational changes which may occur. Examination of homologous aged and non-aged conjugates by this method may yield direct information as to the putative aging-induced conformational change. In the case of a protein for which the three-dimensional structure has already been solved, this may, in principle, be most readily achieved by diffusing suitable OP's into crystals of the native enzyme, to yield modified crystals isomorphous with the solved, native crystal. Initiation of the use of this experimental approach for aged and non-aged OP derivatives of Cht will be described below.

TECHNICAL APPROACH

1. Preparation of OP Ligands for ^{31}P-nmr Studies

Table 1 lists the names and structures of the various OP ligands employed for preparing the corresponding OP-Cht conjugates. Paraoxon was obtained from the Aldrich Chemical Co. (Milwaukee, WI).

Table 1: Structure and Names of OP Ligands and OP Conjugates.

OP Ligands		OP-Cht Conjugate	
Name	Structure	Name	Structure
paraoxon	$(C_2H_5O)_2P(O)(pNP)$	DEP-Cht	$C_2H_5OP(O)(OC_2H_5)Cht$
EIPF	$C_2H_5OP(O)(OisoPr)F$	EIP-Cht	$C_2H_5OP(O)(OisoPr)Cht$
EPinPF	$C_2H_5OP(O)(OPin)F$	EPinP-Cht	$C_2H_5OP(O)(OPin)Cht$
EPDF	$C_2H_5OP(O)F_2$	EP-Cht	$C_2H_5OP(O)(O^-)Cht$
$EP(pNP)_2$	$C_2H_5OP(O)(pNP)_2$	EP-Cht	$C_2H_5OP(O)(O^-Cht$
tabun	$C_2H_5OP(O)[N(CH_3)_2]CN$	EDMP-Cht	$C_2H_5OP(O)[N(CH_3)_2]Cht$

isoPr= isopropyl; Pin= pinacolyl; pNP= p-nitrophenoxy ; CN= cyanide.

1.1. Ethylphosphorodifluoridate (EPDF)

EPDF was prepared by reacting freshly distilled ethyl phosphorodichloridate (Aldrich) with NaF, according to the previously published procedure (25).
^{31}P-nmr (relative to H_3PO_4): δ, -20.4 ppm (t, J_{P-F}, 1007 Hz) (neat). Lit. (26): -20.9 ppm.

1.2. Ethyl isopropylphosphorofluoridate (EIPF)

A solution of isopropanol (2.5 g, 41 mmol) and triethylamine (4.25 g, 42.5 mmol), in 10 ml of absolute ether, was added dropwise to a stirred solution of EPDF (5.0 g, 38.5 mmol) in 25 ml of absolute ether. After 1 hr at room temperature, the reaction mixture was filtered and the ether evaporated. EIPF was purified by distillation (70°C, 20 mm Hg). The pure product was identified by use of ^{31}P-nmr and mass spectrometry:
^{31}P-nmr (relative to H_3PO_4): δ, -10.2 ppm (d, J_{P-F}, 968 Hz) (neat).
MS (EI): 155 (M-15 (CH_3), 86%).

1.3. Ethyl pinacolylphosphorofluoridate (EPinPF)

A solution of pinacolyl alcohol (5.5 g, 54 mmol) and triethylamine (5.5 g, 55 mmol) in 10 ml of absolute ether was added dropwise to a precooled (0-5°C) and stirred solution of EPDF (6.5 g, 50 mmol) in 25 ml absolute ether. Following the addition of the alcohol, the reaction was allowed to proceed at room temperature for an additional 1 hr. The reaction mixture was filtered and flash-evaporated (20°C, 25 mm Hg), and the final product was purified by fractional distillation (50°C, 0.2 mm Hg). The pure product was identified by ^{31}P-nmr and by mass spectrometry:
^{31}P-nmr (relative to H_3PO_4): δ, -9.2 ppm (d.d., J_{P-F}, 952 Hz) (neat)
MS (EI): 156 (M-56 (C_4H_8)), 100%.

1.4. Ethyl bis(p-nitrophenyl)phosphate (EP(pNP)$_2$)

A mixture of p-nitrophenol (7.0 g, 50 mmol) and triethylamine (5.0 g, 50 mmol) in 50 ml of absolute ether was added to a cooled solution (0-5°C) of ethylphosphorodichloridate (Aldrich, 4.0 g, 25 mmol) in 100 ml of absolute ether. After 2 hr at room temperature, the precipitate was filtered and redissolved in a $CHCl_3/H_2O$ mixture (1:1, 100 ml). The organic phase was washed twice with water to remove traces of triethylamine hydrochloride and was then dried over Na_2SO_4. The solid residue obtained after flash evaporation was crystallized from hot ethanol, yielding colorless needle-shaped crystals. m.p., 135°C (27): 132-136°C.
Anal calcd for $C_{14}H_{13}N_2O_8P$: C, 45.64; H, 3.53; N, 7.60.
Found: C, 45.65; H, 3.51; N, 7.34.
^{31}P-nmr (relative to H_3PO_4): δ, -13.4 ppm (S, $CDCl_3$).

In the following, we will describe the synthesis of some novel 2-naphthol-containing OP's for which the numbering will be the same as in the Midterm Report.

1.5. 1-(2-Hydroxynaphthyl)acetamidoethylamine (VI)

Compound VI was prepared by reacting the lactone of 2-hydroxy-1-naphthaleneacetic acid (I) with the corresponding diaminoalkane by the following procedure: The lactone, I (5.0 g, 27 mmol), was dissolved in

70 ml of dry methyl cellosolve. A stoichiometric amount of ethylenediamine (2.2 g) was then added, and the reaction allowed to proceed with stirring for 16 hr at room temperature. The reaction mixture was then added dropwise to 1 liter of anhydrous ether, and the precipitated product (semisolid, oily material) was separated by decantation. The isolated precipitate was further purified by use of a silica gel column onto which it was loaded in methanol and, after washing with $CHCl_3$:CH_3OH (80:20), was finally eluted with CH_3OH. The product, VI, was obtained as a viscous oil.
Anal calcd for $C_{14}H_{16}N_2O_2$, 5% MeOH: C, 67.67; H, 6.69; N, 11.00
Found: C, 67.60; H, 6.61; N, 10.34.
MS (EI): 184 (lactone I, $C_{12}H_8O_2$, 100%), 244 (M^+, 24%).

1.6. 1-(2-hydroxynaphthyl)acetamidobutylamine (XVI)

Compound XVI was prepared like VI, except that ethylenediamine was replaced by 1,4-diaminobutane (3.2 g). Here, too, the product, XVI, was obtained as a viscous oil.
Anal calcd for $C_{16}H_{20}N_2O_2$, 5% MeOH: C, 69.50; H, 7.44; N, 9.92.
Found: C, 69.30; H, 7.48; N, 9.69.
MS (EI): 184 (lactone I, $C_{12}H_8O_2$, 100%), 273 (M+1, 16%).

1.7. O-Ethyl-O-p-nitrophenyl N-(2(2-hydroxynaphthylacetamido)butyl- aminophosphoramidate (E(pNP)BPA, XVII)

XVII was prepared from O-ethyl p-nitrophenylphosphorochloridate (III) and XVI, as follows: A solution of III (3.8 g, 14 mmol) in 20 ml of dry acetonitrile was added dropwise to a solution of XVI (3.8 g, 14 mmol) and 2.5 g triethylamine in 350 ml of dry acetonitrile. After stirring overnight at room temperature, the solvent was removed by roto-evaporation, and the residue was applied to a silica gel column from which it was eluted with $CHCl_3$-CH_3OH (100:5). This procedure resulted in a viscous yellow oil which was rechromatographed in order to remove impurities. The final product was characterized by nmr and ms:
^{31}P-nmr (relative to H_3PO_4): δ, +4.4 ppm ($CDCl_3$).
MS (EI): 501 (M^+, 45%); 483 ((M-18), 100%)

1.8.O-Isopropyl-O-p-nitrophenyl N-(2(2-hydroxynaphthylacetamido))-ethylaminophosphoramidate (IP(pNP)NPA, XV)

O-isopropyl-O-p-nitrophenylphosphorochloridate (XIV) was prepared as follows: A solution of 10 mmol isopropanol and 10 mmol triethylamine in dry ether was added dropwise to a stirred, cold solution (4°C) of 10 mmol of p-nitrophenylphosphorodichloridate (2.5 g, Aldrich) in 10 ml of dry ether. After 15 min, the solution was allowed to warm up to room temperature, and was stirred for an additional 1 hr. The triethylamine hydrochloride which had precipitated was removed by filtration, and the filtrate was evaporated under reduced pressure, using an oil pump (0.2 mm Hg). The residue obtained was characterized by ^{31}P-nmr to demonstrate the presence of only one kind of ^{31}P-nmr signal, and was taken for the next step without further purification. XIV (4 g, 14 mmol), in 20 ml dry acetonitrile, was then added dropwise to a solution of VI (3.5 g, 14 mmol) and 2.5 g triethylamine in 350 ml of dry acetonitrile. Incubation was continued overnight, with stirring, at room temperature. The solvent was removed by roto-evaporation, and the residue was chromatographed two times on a silica gel column (2.5x40 cm) from which it was eluted with $CHCl_3$:CH_3OH (100:5).
Anal calcd for $C_{23}H_{26}N_3O_7P$: C, 56.67; H, 5.34; N, 8.62; P, 6.36.

Found: C, 53.14; H, 5.01; N, 8.10; P, 6.65.
^{31}P-nmr (relative to H_3PO_4): δ, 2.9 ppm ($CDCl_3$)
MS (EI): 487 (M^+, 22%), 488 (M+1, 7%)

1.9. O-isopropyl-O-p-nitrophenyl (2(-hydroxynaphthylacetamido)- butylaminophosphoramidate) (IP(pNP)BPA, XVIII)

XVIII was prepared by reacting XIV and XII as described above for the preparation of IP(pNP)NPA, XV.
^{31}P-nmr (relative to H_3PO_4): δ, +3.3 ppm ($CDCl_3$)
MS (EI): 515 (M^+, $C_{25}H_{30}N_3O_7P$, 25%): 516 (M+1, 8%)

2. Enzymes

2.1. Chymotrypsin

Bovine pancreatic α-Cht (Sigma Type II, 3x crystallized, salt-free and lyophilized), which was employed in the ^{31}P-nmr and optical spectroscopy studies on OP-Cht conjugates,was purchased from Sigma (St. Louis, MO). The active site concentration of solutions of such preparations was determined by use of cinnamoyl imidazole according to Schonbaum et al. (28).

Bovine pancreatic γ-Cht (Sigma Type II, essentially salt-free, 2x crystallized and lyophilized), which was utilized in the preparation of the crystals used to obtain the crystalline OP-Cht conjugates, as well as in the studies in which the conformational stability of OP-Cht conjugates was examined by the transverse urea gradient technique, was also obtained from Sigma (St. Louis, MO).

2.2. Cholinesterase preparations

The dimeric form of AChE from electric organ tissue of Torpedo californica was purified by affinity chromatography, subsequent to solubilization with phosphatidylinositol-specific phospholipase C from Staphylococcus aureus, as described previously (29, 30).

Lyophilized human serum butyrylcholinesterase (BChE, ca. 7% w/w) was purchased from Behring Institute (FRG) and used as such.

2.3. Preparation of OP-Cht conjugates (see also Ref. 18)

Large quantities of OP-Cht (200-400 mg) were obtained by dropwise addition of a fresh, concentrated solution of the appropriate OP, in acetonitrile, to a stirred solution of Cht in 0.1 M Tris, pH 7.8. In no case did the concentration of the organic solvent in the final inhibition medium exceed 5%. The decrease in enzymic activity was monitored spectrophotometrically until >99% inhibition had been achieved. The solution was then dialyzed against double-distilled water, lyophilized, and stored over dessicant at -20°C until employed.

2.4. Reactivation of OP-Cht conjugates

Lyophilized samples of OP-Cht conjugates were dissolved (1-2 μM) in 0.01 M $CaCl_2$-0.1 M Tris, pH 7.8, containing 0.1 M 3-PAM, as described previously (18). At selected time intervals, 5-10 μl aliquots of the reactivation mixture were diluted into 3 ml of 0.01 M $CaCl_2$-0.1 M Tris, pH 7.8, and assayed spectrophotometrically.

2.5. Preparation and characterization of OP-AChE conjugates

Concentrated solutions (ca. 1.5×10^{-2}M) of either pyrenebutyl-OP(O)(OC_2H_5)F (PBEPF) or pyrenebutyl-$OP(O)Cl_2$ (PBPDC)in PBEPF or PBPDC in acetonitrile were added, in small proportions, to solutions (1 mg/ml in 0.1 M NaCl-0.01 M phosphate, pH 7.0) of purified Torpedo AChE, until inhibition of >99% was achieved. The final ratio of moles of OP added per moles of catalytic sites was 3 to 4-fold. Excess OP was removed by gel filtration on a Biogel P-4 column, followed, if necessary, by dialysis.

2.6 Assays

Enzymic activity of AChE was measured essentially according to Ellman et al. (31) using acetylthiocholine as substrate. Reactivation experiments were carried out using 1 mM 2-PAM (Sigma) at pH 7.0.

Assays of Cht activity were performed, according to DelMar et al. (32), using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (1 mM) as substrate.

Protein content of Cht crystals was measured by the Bradford procedure (33).

2.7. ^{31}P-nmr Spectroscopy

^{31}P-nmr spectroscopy at 101.25 MHz was performed using a Bruker WM250 spectrometer. D_2O (20-30%) in the sample tube (10 mm diameter) served for field-frequency locking. A power of 2 W was maintained during data acquisition. The decoupling power was kept low during the delay time (2 s) between accumulations, to avoid internal buildup of heat. Spectral data were accumulated by application of 70°C pulses with a spectral width of 8000 Hz. The temperature was maintained at 20±3°C.

All chemical shifts were recorded from built-in, absolute crystal frequency, and were assigned to the internal standard, hexamethylphosphoramide (HMPA). The chemical shift of HMPA is 30.50 ppm downfield to external 85% phosphoric acid (18). The ^{31}P-nmr spectra of the OP-Cht conjugates and of the model compounds were recorded in a concentration range of 0.8-4 mM, assuming a molecular weight of 25,000 for Cht. From 7 K to 140 K scans were accumulated in each run.

2.8 Transverse urea gradient electrophoresis

This technique was performed essentially as described by Creighton (22), using acrylamide slab gels in which the urea concentration varied linearly from 0 to 8 M across the slab in the direction parallel to electrophoretic migration. The gels were made by polymerizing gels in which the urea gradient had been formed by mixing two acrylamide solutions containing the two limiting concentrations of urea. A slight reverse gradient (from 15%

to 11%) of acrylamide was employed in order to compensate for the effect of urea on the electrophoretic properties of proteins which did not involve unfolding. The buffer employed was 0.05 M Tris-acetate, pH 4.0. Methyl green was employed as the electrophoresis marker, and ca. 400 μl of a solution of protein concentration 1.3 mg/ml were applied to the gel. Gels were stained with Coomassie Brilliant Blue R-250.

2.9. Optical spectroscopy

Circular dichroism (CD) spectra were carried out using a JASCO J-500C spectropolarimeter. The absorption anisotropy factor, g_{ab}, is defined by $g_{ab} = (\varepsilon_l - \varepsilon_r)/\varepsilon$, where ε_l and ε_r are the extinction coefficients for left- and right-handed circularly polarized light, respectively, and ε is their average (34).

Absorption spectra were measured in a Hewlett-Packard Diode Array double-beam spectrophotometer, and fluorescence measurements in a Shimadzu RF-540 spectrofluorometer.

2.10. Crystallization of γ-Cht and preparation of crystalline OP-Cht conjugates

γ-Cht was crystallized essentially according to Segal et al. (35) from concentrated $(NH_4)_2SO_4$ in 0.02 M sodium cacodylate, pH 5.6, at room temperature. Well-formed tetragonal crystals appeared within a month, and crystals of suitable size, i.e. 0.2x0.2x0.6 mm, were employed either directly for crystallographic measurements or for chemical modification.

Crystals of DEP-Cht were prepared by prolonged soaking of crystals in a solution of of 0.4 mM paraoxon in 65% saturated $(NH_4)_2SO_4$, 0.4% dioxane, 0.01 M sodium cacodylate, at room temperature, with weekly refreshing of the inhibitor solution. The extent of inhibition was assessed by dissolving crystals in 0.0025 N HCl, and assaying the solution for Cht activity and protein content as compared to control crystals.

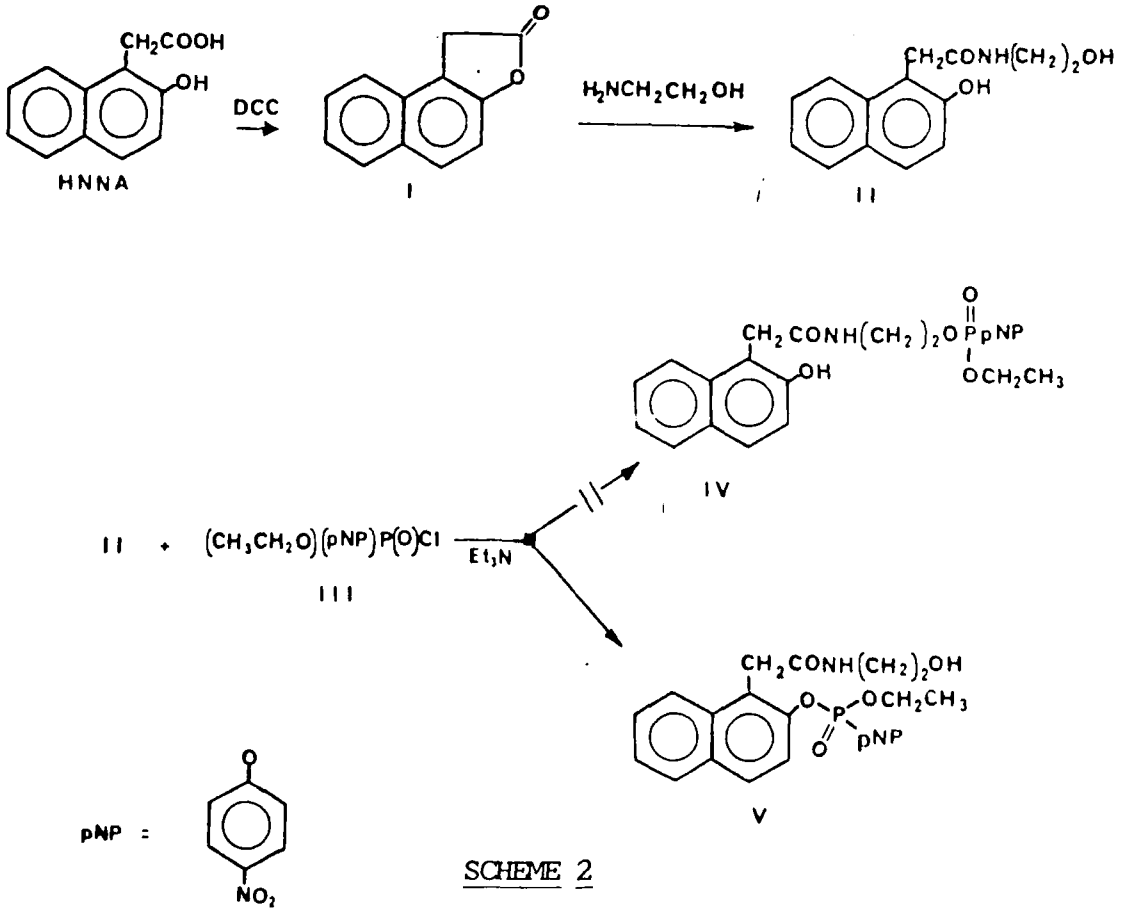
Crystals of IP-Cht and EP-Cht, i.e., monoisopropylphosphoryl- and monoethylphosphoryl-Cht, respectively, were similarly prepared, except that paraoxon was replaced by 10 mM diisopropylphosphorofluoridate and 10 mM ethyl isopropylphosphorofluoridate, respectively.

RESULTS AND DISCUSSION

1. Synthesis and Anti-ChE Properties of Novel 2-Naphthol-Containing Organophosphates

1.1. Synthesis

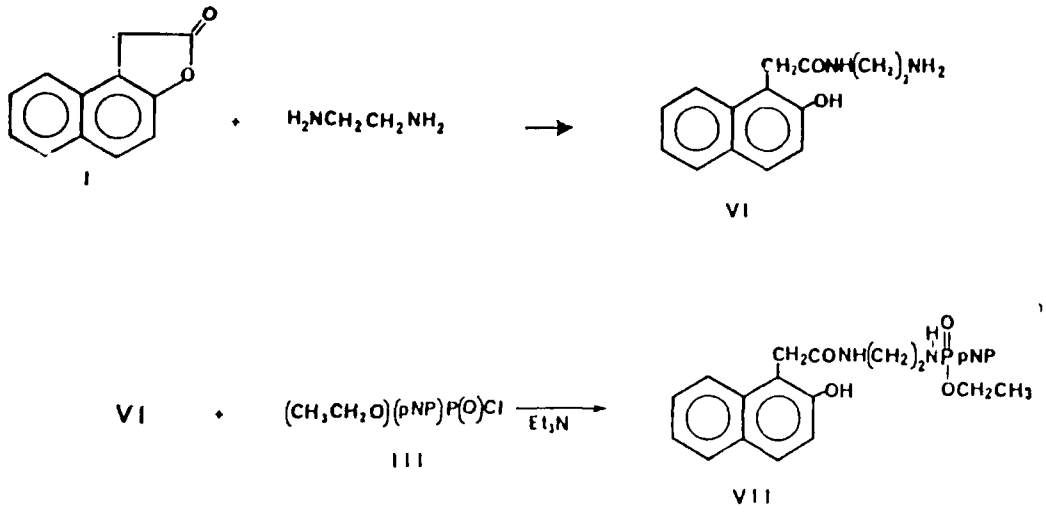
In the grant application, we proposed to embark on the synthesis of a new family of fluorescent OP's in which the fluorophore would be 2-naphthol; this choice was based on the reported spectroscopic properties of probes based on aromatic hydroxyl derivatives, which exhibit excited state ionization constants very different from those observed in the ground state (36), a fact which is reflected in their fluorescence. Thus the rate of proton transfer which such probes undergo is sensitive to the availability of proton acceptors and donors, as well as to their pK_a; their fluorescence, in protein conjugates, should be highly conformation-sensitive (20) and particularly suitable for detecting the putative conformational changes involved in aging. The synthetic approach which we have adopted is based on the above-mentioned work of Laws et al. (20), in which the lactone of 2-hydroxy-1-naphthalene acetic acid (HNAA) was used as a reagent for attachment of the 2-naphthol probe to proteins. In our case, HNAA was initially used in a coupling reaction with an appropriate amino alcohol sidearm, to yield an adduct which could then be coupled with a suitable activated OP. Indeed, in the initial phase of this project HNAA was coupled, via an aminoethanol extension arm (II), to a phosphorochloridate (III), to obtain the desired fluorescent OP (IV), as shown in Scheme 2.



SCHEME 2

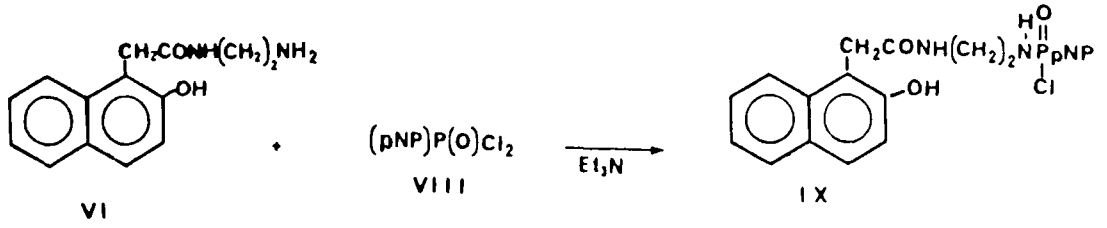
However, as is also depicted in Scheme 2, reaction occurred preferentially

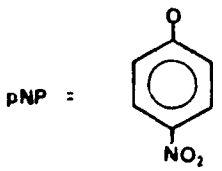
with the aromatic hydroxyl group, leading to formation of an O-aryl ester (V), rather than to the expected ligand (IV). So as to obtain a suitable naphthol-containing OP of the general type represented by IV, we decided to employ ethylenediamine as the sidearm. We hoped to thus direct phosphorylation to the amino group (Scheme 3, VI), since it is a stronger nucleophile than the aromatic hydroxyl residue. This reaction (Scheme 3) resulted in an almost exclusive regioselective phosphorylation, to yield the desired product ,VII.



SCHEME 3

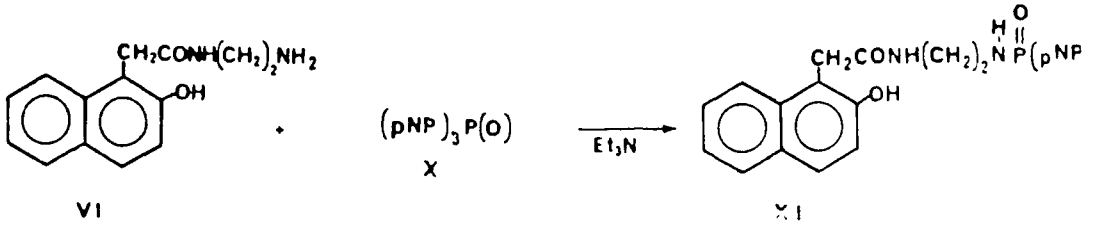
Compound VII, O-ethyl O-p-nitrophenyl N-{2(2-hydroxynaphthyl-acetamido)ethylamino°phosphoramidate (E(pNP)NPA), was expected to form a non-aged conjugate with AChE and other serine hydrolases. Before proceeding to a detailed study of the kinetics and stoichiometry of the inhibition process, we concentrated on the synthesis of the appropriate OP which could be used for production of the homologous aged OP-enzyme conjugates. The rationale for this approach was the known observation that OP conjugates containing secondary alkyl substituents age more rapidly than those containing primary substituents (1). Our first attempt was to prepare the chloridate analog of E(pNP)NPA (IX), as depicted in Scheme 4.

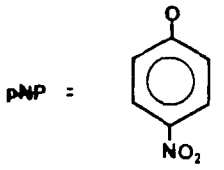




SCHEME 4

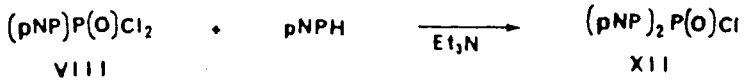
Separation of IX from the reaction mixture proved difficult since the chloridate is very sensitive to hydrolysis. To overcome this problem, we decided to replace the chloride by the p-nitrophenoxy (pNP) group. and, thereby, to obtain bis-(O-p-nitrophenyl) N-{2(2-hydroxynaphthyl-acetamido)ethylamino°phosphoramidate ($(pNP)_2$NPA) (XI). We attempted to obtain this compound by the procedure outlined in Scheme 5.

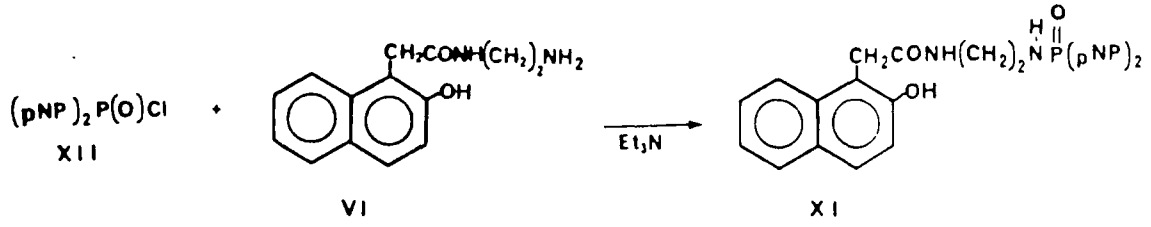


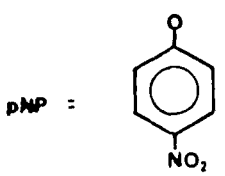


SCHEME 5

The yield of the desired product (XI) obtained by this procedure was not satisfactory. Another synthetic approach was, therefore, adopted. This involved synthesis of bis-(p-nitrophenyl)phosphorochloridate (XII), and its reaction with VI to yield the expected product, XI, as shown in Scheme 6.





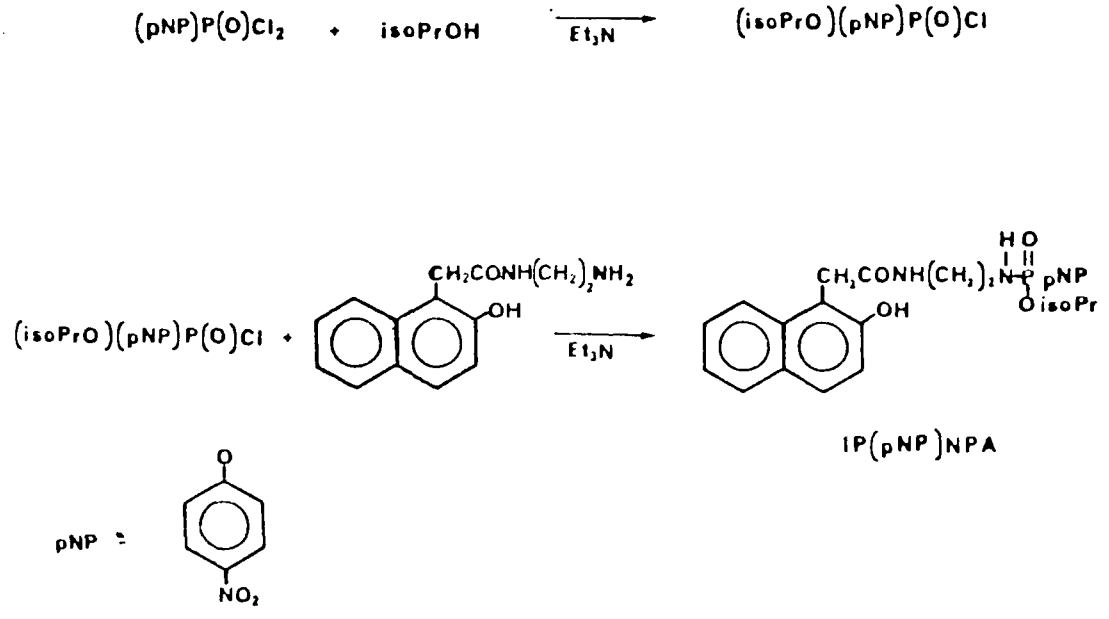


SCHEME 6

Due to the instability of the intermediate, XII, it was not isolated from the reaction mixture; instead, after the first reaction had gone to completion, as indicated by monitoring its progress by ^{31}P-nmr, one equivalent of VI was added to the reaction mixture.

The hydrolytic stabilities of both naphthol-containing OP's, viz. E(pNP)NPA (VII) and $(pNP)_2$NPA (XI), were found to be unsatisfactory, and this necessitated frequent reprocessing. Extraction of free p-nitrophenol, which was identified as the main contaminant, into carbonate buffer (pH 10) resulted in significant reduction in the amount of free p-nitrophenol. E(pNP)NPA released 1±0.1 equivalents of p-nitrophenol upon treatment with 1 N NaOH. $(pNP)_2$NPA, as expected, displayed a biphasic profile for release of p-nitrophenol, corresponding to a total release of approximately two moles of p-nitrophenol per mole of ligand.

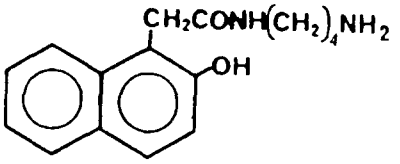
Despite various attempts described thus far to prepare 2-naphthol-containing OP's suitable for obtaining aged 2-naphthol-containing OP conjugates of serine hydrolases, the OP's obtained so far were of a degree of purity inadequate for kinetic and physicochemical studies. Thus, we were unsuccessful in our attempts to obtain satisfactory preparations of either $(pNP)_2$PA (XI) (see Scheme 5) or of its P-Cl analog (IX, see Scheme 4), which was also designed to produce the corresponding aged OP-AChE conjugate. In order, nevertheless, to obtain an aged naphthol-containing OP conjugate homologous to the non-aged OP-AChE conjugate, we decided to adopt the following synthetic route (Scheme 7), to obtain a product, IP(pNP)NPA (XV), which might be more stable and amenable to purification.



SCHEME 7

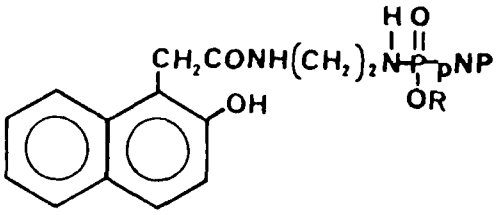
It was predicted that the O-linked isopropyl group would be detached from the phosphorus atom in the corresponding OP-enzyme conjugate, thus producing the desired aged conjugate. The procedure envisaged for preparation of IP(pNP)NPA (XV) was similar to that used to obtain VII (Scheme 3). After a number of modifications of the proposed synthetic route (see Technical Approach) we were able to obtain IP(pNP)NPA with a satisfactory degree of purity. As in the case of E(pNP)NPA (VII), the homogeneous product displayed nmr (^{31}P, ^{1}H) and ms spectra consistent with the proposed structure. Its excitation and emission spectra in aqueous solution were almost identical to that of the O-ethyl homolog, E(pNP)NPA, as were their pH-dependences. The spectroscopic characteristics of both compounds are consistent with the presence of a 2-naphthol residue. IP(pNP)NPA was 3-fold more sensitive to alkaline hydrolysis (0.1 N NaOH) than E(pNP)NPA. This observation is consistent with the hydrolytic-stability rank order among OP esters. Also, approximately one equivalent of p-nitrophenol was released upon hydrolysis.

In order to expand the family of 2-naphthol-containing OP conjugates, we attempted to obtain homologous ligands in which the 2-naphthol residue is separated from the phosphorus atom by a spacer containing more than two methylene groups. To this end, we have synthesized 1-(2-hydroxynaphthyl)acetamidobutylamine (XVI):



XVI

XVI was prepared by reacting 1,4-diaminobutane with the lactone of HNAA (I) in a manner analogous to that employed for the synthesis of VI (see Scheme 3). During the synthesis of XVI, the procedure adopted from Scheme 3 was slightly modified. XVI was further reacted with pNP-OP(O)(OR)Cl (R = C_2H_5, C_3H_7) as outlined in the analogous syntheses of VII and XV in Schemes 3 and 7, respectively. We hoped thus to obtain a pair of ethyl (XVII) and isopropyl-containing OP ligands in which the naphthyl group would be separated by a four methylene spacer from the phosphorus atom.



XVII (R = C_2H_5); XVIII (R = C_3H_7)

Indeed, towards the end of this project, we were able to obtain both XVII and XVIII with a satisfactory degree of purity. These products displayed ^{31}P-nmr, ms and fluorescence spectra consistent with their proposed structures.

1.2. Inhibition of cholinesterases by 2-hydroxynaphthyl-containing organophosphates

First-order plots of data obtained for the inhibition of Torpedo AChE or of human BChE by a large excess of either E(pNP)NPA or IP(pNP)NPA provided straight lines which passed through the origin. However, a plot of the first-order rate constants vs. the concentrations of the OP inhibitors, in the range of 10-100 μM, revealed saturation curves which indicated, in both cases, formation of a pre-complex prior to the phosphorylation step. We thus assumed that the following steps are involved in the kinetics of the inhibition:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k'} E'$$

SCHEME 8

where E and I are the enzyme and inhibitor, respectively; EI and E' denote, respectively, the reversible complex formed prior to phosphorylation and the phosphorylated enzyme; and K_I and k' represent the dissociation constant for EI and the unimolecular rate constant for phosphorylation of the enzyme, respectively. The mathematical solution for Scheme 8 is given in Eq. 1:

$$1/k_{obs} = (K_I/k')(1/I) + 1/k' \qquad (1)$$

The double-reciprocal plots ($1/k_{obs}$ vs. 1/I), plotted according to Eq. 1, provided straight lines (see Figs. 1 and 2) from which K_I and k' were calculated for the inhibition of AChE and BChE by E(pNP)NPA and by IP(pNP)NPA. The bimolecular rate constant is given by $k_i = k'/K_I$. The kinetic constants obtained in accordance with the above-mentioned scheme are given below in Table 2:

Table 2: Inhibition Rate Constants for the Reaction of 2-Hydroxynaphtol-Containing OPs and ChEs (0.05 M Phosphate, pH 8.0, 25C).

Enzyme	OP	K_I(mM)	k'(min^{-1})	k_i($M^{-1}min^{-1}$)
AChE, Torpedo	E(pNP)NPA	0.212	0.040	188
	IP(pNP)NPA	0.355	0.025	70
BChE, human	E(pNP)NPA	0.0058	0.10	1.74×10^4
	IP(pNP)NPA	0.0085	0.10	1.17×10^4

It should be noted that the rate of inhibition was affected by the presence of the acetonitrile which was required in order to prepare stock solutions of the inhibitor. Thus, the inhibition rate constant decreased 2-fold over a concentration range of 0.5-2% acetonitrile. The results presented in Table 2 were obtained in the presence of 1% acetonitrile. The data obtained suggest that BChE can accommodate the 2-naphthol-containing OP's in its active site much more comfortably than AChE. However, despite the low bimolecular rate constant obtained for the inhibition of AChE by either E(pNP)NPA or IP(pNP)NPA, a relatively high proportion of the total AChE activity could be inhibited by using a high concentration of OP and a long incubation period (24-48 hr). Indeed, preliminary experiments, in which inhibition was followed by gel filtration and extensive dialysis, to remove the excess of unreacted ligand, showed the presence of protein-bound ligand as judged by the excitation and emission spectra at neutral and alkaline pH. The inhibited enzyme conjugates, ENPA-AChE and ENPA-BChE, which were obtained by use of E(pNP)NPA, could not be reactivated by 2-PAM, by TMB4 or by F^-. However, spectroscopic data, obtained after gel filtration of the reactivation mixtures, indicate that this resistance to reactivation is not due to detachment of the fluorophore from the conjugate. It should be noted that the 2-naphthol-containing OP's belong, like tabun, to the P-N family, which yield OP-ChE conjugates which are known to be highly resistant to reactivation (37, 38).

In order to further study the nature of the 2-naphthol-containing conjugates of Torpedo AChE, we studied the interaction of diisopropylphosphorofluoridate-inhibited AChE with E(pNP)NPA. To our surprise, it was found that a substantial amount of fluorescence was incorporated into the enzyme which had been pre-blocked with DFP. It is worth noting that we have also found (see below) that more than one mole of the pyrene-containing OP ligand PBPDC can react with Torpedo AChE, and that we have also obtained evidence for more than one site of interaction of pinacolyl-containing pyrenebutyl organophosphates with this enzyme.

Due to the uncertainty which we encountered with respect to the stoichiometry of the naphthol-containing OP-AChE conjugates, we decided to postpone further work with them until this issue has been clarified. (See also discussion at the end of the following section.)

2. Conformational Stability of Aged and Non-Aged Organophosphoryl Conjugates of Chymotrypsin and Acetylcholinesterase.

During the course of this project we obtained detailed experimental results, using the technique of circular dichroism (CD), in which we compared the conformational stability of two homologous pairs of non-aged and aged conjugates of Cht, namely, PBEP-Cht and PBP-Cht, and DEP-Cht and EP-Cht. The rationale behind these experiments was, in brief, that the resistance to reactivation displayed by aged OP conjugates might be the result of the formation of a salt bridge or hydrogen bond as a result of the aging reaction, e.g., between the newly formed negative charge on the OP group and the active-site histidine, which, in turn, could lead to stabilization of the structure of the protein; this might find expression in increased resistance to denaturation. Evidence for such a stabilization was presented by Masson and Goasdue (21), who used the transverse urea gradient technique of Creighton (22) to demonstrate an increased resistance to unfolding of an aged OP conjugate of human serum cholinesterase.

We studied the CD spectrum, as a function of Gu.HCl concentration of the two homologous pairs of aged and non-aged conjugates of Cht mentioned above. In both cases, we were able to clearly show that the aged conjugate was significantly more stable to unfolding than its corresponding non-aged conjugate. Moreover, in the case of the pair of pyrene-containing conjugates, we were able to show that the pyrene group in the non-aged conjugate is oriented differently than the corresponding group in the aged conjugate, and appears to undergo more reorientations upon unfolding. Th principal conclusions from these data can be discerned from Fig. 3, in which we briefly summarize the principal finding by plotting the absorption anisotropy factor at 230 nm, g_{230}, as a function of Gu.HCl concentration, for the two pairs of conjugates. At this wavelength, absorption anisotropy can be attributed primarily to the polypeptide backbone. It can be clearly seen that both non-aged conjugates unfold at about the same Gu.HCl concentration, and that, in both cases, aging stabilizes resistance to unfolding to about the same extent.

We have since compared DEP-Cht and EP-Cht by the same technique used by Masson and Goasdue (21), namely, the transverse urea gradient technique of Creighton (22), using a 0-8 M urea gradient. Typical electrophoretograms obtained by use of this technique are shown in Fig. 4. When studied at $4^{o}C$ or at room temperature, DEP-Cht unfolded, under the experimental conditions employed, at ca. 5.5 M and 4.9 M urea, respectively. Under these two same sets of conditions, no transition could be detected for the aged conjugate, EP-Cht. When, however, the temperature of electrophoresis was raised to $37^{o}C$, the urea concentration at which DEP-Cht unfolded was lowered to 4.3 M, and EP-Cht unfolded at 5.6 M urea. From the midpoint and slope of the unfolding curve on the gel, one can gain an estimate of the unfolding energies involved (39). It thus appears that the aged conjugate is stabilized by ca. 2 kcal relative to the non-aged conjugate. This conclusion is further supported by a recent nmr study of Anderson et al. (40) who calculated that a single salt bridge stabilizes the native state of lysozyme by 3-5 kcal/mole. It is difficult to calculate a similar figure for the CD measurements carried out as a function of Gu.HCl concentration without much more detailed measurements (41), but here, too, values of a few kilocalories can be estimated. Thus, the involvement of one, or a small number of salt bridges or hydrogen bonds in the aging-induced stabilization is very plausible. Similar conformational stabilization due to the insertion of a $P\text{-}O^{-}$ bond into the active site of AChE purified from fetal bovine serum was recently demonstrated by one of us, utilizing a monoclonal antibody which had been raised against DIP-AChE (42).

It was decided to extend our studies of conformational stability to an homologous pair of OP conjugates of AChE. For this purpose we used the dimeric form of AChE from electric organ tissue of Torpedo californica, purified by affinity chromatography subsequent to solubilization with phosphatidylinositol-specific phospholipase C (29, 30). Aged and non-aged conjugates were prepared, as reported previously for both for electric eel AChE (16) and for Cht (17), by use of PBPDC and PBEPF, respectively, to yield PBP-AChE and PBEP-AChE (for details see Technical Approach). All the experiments described below were carried out using the conjugates of Torpedo AChE.

The extinction coefficient of the bound pyrenebutyl moiety in PBEP-AChE was found to be $\varepsilon_{347} = 2.4 \times 10^{4} M^{-1} cm^{-1}$, while that of free PBEPF at this wavelength is only $1.9 \times 10^{4} M^{-1} cm^{-1}$. The stoichiometry of the non-aged

conjugate could thus be calculated to be 0.94 mole of OP per mole of active sites. The extinction coefficient of the bound pyrenebutyl moiety in the aged conjugate, PBP-AChE, could, in principle, be determined with a suitable pyrene-containing OP, such as $PBP(pNP)_2$, which, unlike PBPDC, does not undergo rapid hydrolysis. However, this OP was not available at the time these experiments were performed.

The specificity of binding of PBEPF and PBPDC to the active site serine of the Torpedo AChE was examined by exposing an AChE preparation, the active sites of which had been completely blocked by prior exposure to either paraoxon or DFP, to PBEPF or PBPDC, under the same experimental conditions that were routinely used for preparation of PBEP- or PBP-AChE, respectively. After removal of the excess pyrenebutyl-containing OP by gel filtration and dialysis, the fluorescence spectrum was measured and compared to that of native enzyme which had undergone the same treatment. The binding of PBEPF to AChE was found to be specific, the fluorescent quantum yield ratio of the control enzyme being less than 2% of that of the conjugate. Binding of PBPDC to AChE was found, however, under the experimental conditions employed, to involve a non-specific component, the corresponding figure being ca. 20%. This lack of specificity is, presumably, due to the high reactivity of PBPDC. In interpreting our present spectroscopic measurements it should, therefore, be borne in mind that PBP-AChE does not contain a completely homogeneous population of the pyrenebutyl probe. Since only a 3-fold molar excess of PBPDC over AChE catalytic sites was employed, it is unlikely that this inhomogeneity is substantial; due, however, to the uncertainty with respect to the quantum yields of the specific and nonspecific bound pyrenes the extent of nonspecific labelling cannot be unequivocally established.

The fluorescence quantum yield of PBP-AChE was found to be ca. 20% lower than that of PBEP-AChE upon excitation at 347 nm. Their conformation, as reflected by CD measurements, is similar, although not identical (compare the CD spectra obtained at zero guanidine hydrochloride in Figs. 5 and 6).

The relative stability to denaturation of the two pyrenebutyl-containing AChE conjugates was studied, as already had been done for the corresponding Cht conjugates, by monitoring their CD spectra as a function of the concentration of Gu.HCl. The results of these measurements are presented in Figs. 5 and 6. The dependence of the absorption anisotropy factor (g_{ab}) at 230 nm, g_{230}, on the Gu.HCl concentration is shown in Fig. 7. The midpoint of the conformational transition is at 0.6 M Gu.HCl for the non-aged conjugate, PBEP-AChE, and at 1.2 M Gu.HCl for its aged counterpart, PBP-AChE. Inspection of the corresponding curves for PBEP-Cht and PBP-Cht, presented above in Fig. 3, shows that the midpoints of unfolding for these two conjugates are 2.7 M and 3.7 M Gu.HCl, respectively. Thus, although unfolding of the OP-AChE conjugates occurs at lower Gu.HCl concentrations than for the OP-Cht conjugates, the results are qualitatively similar, and indicate that the negative charge introduced by dealkylation imparts a similar degree of stabilization in the two systems.

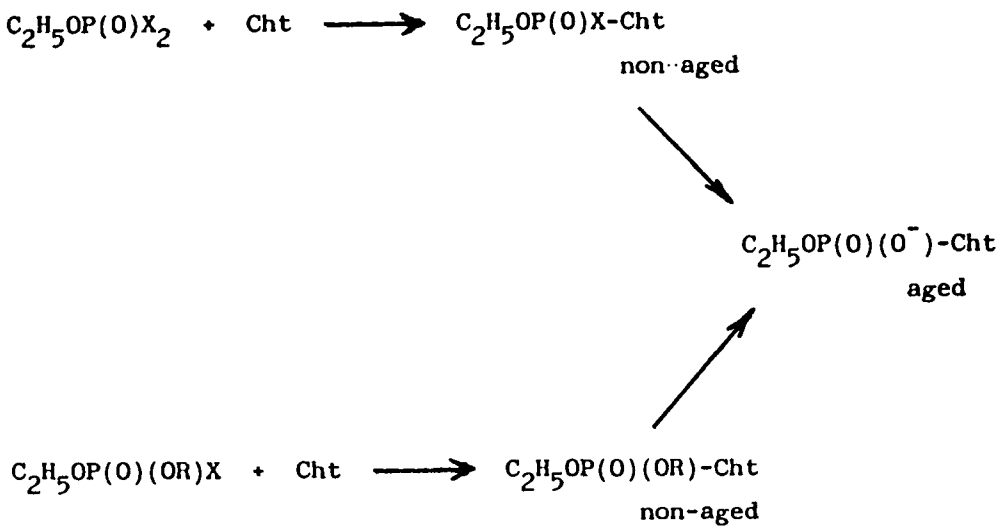
In conclusion of this section, we would like to point out, once more, the uncertainty introduced into the data obtained for AChE by the lack of stoichiometry encountered in the case of the aged conjugate, PBP-AChE. The possibility that PBPDC is reacting with the enzyme at an additional site bears further exploration, in particular due to the observation of an additional alkylation site in AChE from Torpedo nobiliana by Cohen and

coworkers (43) and our own unpublished results with pyrenebutylpinacolyl containing OP-AChE conjugates. It should also be noted that the free sulfhydryl group of Cys231 in AChE from Torpedo californica may also serve as a putative alkylation site, since a varied repertoire of sulfhydryl reagents can interact with this group with concomitant inhibition of enzymic activity (N. Steinberg, E. Roth and I. Silman, unpublished). These considerations might also be pertinent to the similar lack of specificity encountered in use of our novel naphthol-containing OP's, as was discussed above.

3. ^{31}P-nmr Spectroscopy of OP-Cht Conjugates

3.1. Rationale

^{31}P-nmr has been demonstrated by us (18) and by others (23, 24) to be applicable to characterization and comparison of aged and non-aged phosphorylated derivatives of serine hydrolases such as Cht and trypsin. In a recent report, for example (18), we utilized pyrenebutyl-containing OP inhibitors for preparation of homologous pairs of aged and non-aged conjugates of Cht. The relative chemical shifts of the non-aged triester, pyrenebutyl-OP(O)(OC_2H_5)-Cht (PBEP-Cht) and the corresponding aged diester, pyrenebutyl-OP(O)(O^-)-Cht (PBP-Cht), were found to be in good agreement with the corresponding ^{31}P-nmr chemical shifts of model compounds. During this research project (which was conducted with the support of USAMRDC Grant No. DAMD17-83-G-9548), we further observed that OP-Cht conjugates obtained by use of either pyrenebutyl-OP(O)(O-pinacolyl)Cl (PBPinPC) or its fluoro analog, PBPinPF, displayed similar ^{31}P-nmr chemical shifts. Prolonged incubation of the corresponding pinacolyl-containing Cht conjugate, pyrenebutyl-OP(O)(O-Pinacolyl)-Cht (PBPinPCht), resulted in the gradual appearance of a second ^{31}P-nmr signal downfield from the signal obtained with a fresh pinacolyl-containing OP-Cht solution. The most likely explanation is that the pinacolyl residue has been removed, presumably with concomitant formation of a P-O^- function, so as to produce the aged form, PBP-Cht. However, although the ^{31}P-nmr chemical shift of the time-dependent signal was consistent with the formation of a P-O^- bond, the chemical shift observed was approximately 0.4-0.5 ppm upfield relative to aged PBP-Cht conjugates which were obtained from either pyrenebutyl-OP(O)Cl_2 (PBPDC), pyrenebutyl-OP(O)(p-nitrophenoxy)$_2$ (PBP(pNP)$_2$) or pyrenebutyl-OP(O)(p-nitrophenoxy)Cl (PBP(pNP)C). All three OP ligands produced the aged form via hydrolysis of an unstable OP-Cht intermediate (see scheme below). In the original proposal for the present project, we speculated that, while the reasons are not yet clear for the differences in the chemical shifts observed for PBP-Cht obtained by different chemical routes, one may assume that the aging of PBPinP-Cht is accompanied by chemical changes at or adjacent to the active site of the inhibited enzyme (e.g., alkylation of an amino acid residue). Such a reaction might well perturb the ^{31}P-nmr chemical shift of the aged conjugate. We proposed, therefore, to investigate this possibility by use of a second series of OP ligands, which were expected to produce the aged enzyme conjugate either by dealkylation or by hydrolysis. The following scheme depicts such alternative routes for obtaining the aged conjugate, C_2H_5OP(O)(O^-)Cht:



where X= F, p-nitrophenoxy and R= ethyl, isopropyl, pinacolyl.

SCHEME 9

It was hoped that observation of a consistent pattern of differences between the chemical shifts of the the aged conjugates obtained by different routes (i.e., hydrolysis vs. dealkylation) would help to clarify whether a chemical change in the enzyme indeed occurred during aging via the dealkylation route. To this end, we prepared the aged conjugate, $C_2H_5OP(O)(O^-)$Cht, by using five different OP ligands, two of which produced the aged conjugate by hydrolysis of an unstable intermediate (see Scheme 9) and three in which aging was assumed to occur via a mechanism which involved removal of an alkyl group from a Cht-bound OP residue. The names and structures of the OP ligands used to prepare the corresponding OP-Cht conjugates for the ^{31}P-nmr studies were already summarized above in Table 1. ^{15}N-nmr studies have recently shown (44, 45) that some boronic acids and boronic acid peptides form adducts with the active-site histidine rather than with the serine. Accordingly, ethyl (N,N-dimethylamido)phosphorocyanidate (tabun) was used to obtain the corresponding P-N analog of the C_2H_5O-containing conjugate, to be able to use ^{31}P-nmr chemical shifts to assess the possible involvement of P-N bonds in aging of conjugates obtained from the appropriate parent OP's.

3.2. Correlation between ^{31}P-nmr and reactivatibility of OP-Cht conjugates

^{31}P-nmr chemical shifts were determined relative to hexamethylphosphoramide (HMPA) as internal standard under experimental conditions which we had described earlier (18). To correlate the chemical shifts of the aged and non-aged conjugates with various substituents attached to the P atom, we established the ^{31}P-nmr chemical shifts of model compounds under the same experimental conditions which were employed for the OP-Cht conjugates. Results of ^{31}P-nmr measurements are summarized in Table 3.

Table 3: ^{31}P-nmr Chemical Shifts (δ , ppm relative to HMPA) of native and denatured OP-Cht Conjugates at pH 7.0 (0.1 M Tris)[a].

OP-Cht conjugate	OP[b] used	, ppm (from HMPA)[c] native	denatured[d]
$(C_2H_5O)_2P(O)$Cht (non-aged)	paraoxon	-28.84	-31.07
$C_2H_5OP(O)$(OisoPr)Cht (non-aged)	EIPF	-29.55	-31.83
$C_2H_5OP(O)$(OPin)Cht (non-aged)	EPinPF	-30.07	-31.72
$C_2H_5OP(O)[N(CH_3)_2]$Cht (aged)	tabun	-15.84	-17.39;-17.69
$C_2H_5OP(O)(O^-)$Cht (aged)	EPDF	-27.70	-29.27
	$EP(pNP)_2$	-27.68	-29.28
	paraoxon	-27.72[e]	-29.28[e]
		-27.95[e]	
		-28.08[e]	
	EIPF	-27.70[e]	-29.36[e]
		-27.95[e]	-30.24[e]
		-28.07[e]	
	EPinPF	-27.68[e]	-29.27[e]
		-28.23[e]	-29.52[e]
		-28.39[e]	
model compounds:			
$(C_2H_5O)_3P(O)$	TEP	-30.38[f]	
$(C_2H_5O)_2P(O)OH$	DEPA	-29.20	
$C_2H_5OP(O)$(OisoPr)OH	EIPA[g]	-30.06	-29.89
$C_2H_5OP(O)$(OPin)OH	EPinPA[g]	-29.67	-29.47
$C_2H_5OP(O)(OH)_2$	MEPA	-27.95	
$C_2H_5OP(O)[N(CH_3)_2](OH)$	EDMPA	-18.54	

a. Results are average of 2-4 measurements. Estimated error: 0.05 ppm.
b. For definitions see Table 1. c. Negative signs indicate an upfield shift relative to HMPA. d. 6 M guanidine.HCl. e. Signals observed following dialysis of samples which were incubated at 38°C for 120 hr.
f. from ref. 18g. Obtained by hydrolysis of either EIPF or EPinPA or tabun in 0.1 NaOH.

As demonstrated previously by van der Drift et al. (24) and by Grunwald et al. (18), replacing one alkoxy group in a phosphate triester by a negatively charged oxygen, to form a diester, produced a significant downfield shift (ca. 1.2 ppm) relative to the triester. The chemical shift is further displaced downfield upon insertion of a second ionized oxygen (e.g., $(C_2H_5O)_2P(O)(O^-)$ to $C_2H_5OP(O)(O^-)_2$). Replacing the alkoxy residue with an N,N-dimethylamido group caused an even higher downfield shift (ca. 14 ppm) in the ^{31}P-nmr signal. A similar downfie d was observed by us upon replacement of one methoxy group in trimethylphosphate - $((CH_3O)_3P(O)$, -21.47 ppm - by an N,N-dimethylamido group - $(CH_3)_2NP(O)(OCH_3)_2$, -14.57 ppm. When OP model compounds were transferred from 0.1 M Tris buffer, pH 7.0, to 6 M guanidine hydrochloride (Gu.HCl), pH 7.0, the ^{31}P-nmr shifts moved ca. 0.2 ppm downfield, while maintaining their relative positions prior to addition of the 6 M Gu.HCl. The ^{31}P-nmr chemical shifts of the diesters, ethyl isopropylphosphoric acid and ethyl pinacolylphosphoric acid, were found to be upfield, relative to diethylphosphoric acid, a finding which is consistent with similar differences observed in the chemical shifts of branched vs. unbranched trialklyphosphates (18, 24, 46). These results lend support to the overall approach of using ^{31}P-nmr spectroscopy to study the OP moiety of OP-Cht conjugates. The triester OP conjugates, DEP-, EIP- and EPinP-Cht could be partially reactivated in the presence of 0.1 M 3-PAM (Table 4), and could, accordingly, be defined as non-aged conjugates. In contrast, and as expected, EP-Cht obtained by use of either EPDF or $EP(pNP)_2$ could not be reactivated, irrespective of the inhibitor employed. Thus, aging was obtained by either instantaneous hydrolysis (removal of fluoride) or gradual detachment of the p-nitrophenoxy residue (see Scheme 9). When either DEP-, EIP- or EPinP-Cht was

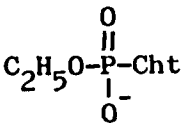
Table 4: Percent Reactivation of OP-Cht Conjugates By 0.1 M 3-PAM at 25C.

OP-Cht[a] Conjugate	pH 7.8[b]		pH 10.0[c]	
	48 hr	max.[d] reactiv.	48 hr	max[e] reactiv.
$(C_2H_5O)_2P(O)$-Cht	62	77	nm[f]	nm
$(C_2H_5O)P(O)$(OisoPr)-Cht	47	64	27	69
$(C_2H_5O)P(O)$(OPin)-Cht	73	95	49	83
$(C_2H_5O)P(O)(O^-)$-Cht	<2	<2	<2	<2
$(C_2H_5O)[(CH_3)_2N]P(O)$-Cht	<2	<2	<2	<2

a. 1-2 uM OP-Cht conjugate. b. 0.1 M tris c. 0.1 M carbonate. d. determined after 96 hr. e. determined after 150 hr. f. not measured

incubated at 38°C for 120 hr, approximately 75-90% aging occurred; i.e., 0.1 M 3-PAM could restore only 10-25% of the activity obtainable from fresh solutions of the corresponding conjugates.

Conversion of the reactivatable OP-Cht conjugates into the aged forms, under the above experimental conditions, was accompanied by disappearance of the ^{31}P-nmr signal of the non-aged OP-Cht conjugate, with concomitant appearance of a downfield chemical shift signal (at -27.68 - -27.70 ppm relative to HMPA; Fig. 8) which is consistent with the formation of a diester OP moiety, presumably $C_2H_5OP(O)(O^-)$-Cht (EP-Cht). The observation that the aged form was obtained by use of five different OP ligands, and via two different chemical routes, strongly supports the suggested structure of the aged conjugate (see Scheme 10).



SCHEME 10

We earlier used a similar approach (18) to establish the presence of a P-O$^-$ bond in pyrene-containing aged OP conjugates of Cht. Our conclusion is also consistent with the ^{31}P-nmr measurements of van der Drift et al. (24) who reported the presence of a P-O$^-$ function in isoPrOP(O)(O$^-$) conjugates of various serine hydrolases. Although DEP-Cht, EIP-Cht and EPinP-Cht were demonstrated to form an aged conjugate with a ^{31}P-nmr chemical shift signal similar to that obtained by use of EPDF or EP(pNP)$_2$, additional peaks of relatively high intensity were observed in the ^{31}P-nmr spectra of OP-Cht conjugates in which aging had been thermally induced. None of these peaks (-27.95 - -28.40 ppm relative to HMPA; see Table 3 and Fig. 8) could be ascribed to an unfolded form of the corresponding conjugate. It should also be noted that the nmr spectra were recorded following extensive dialysis to remove low-molecular-weight components. Furthermore, EP-Cht preparations obtained by use of either EPDF or EP(pNP)$_2$ appeared to be heat-stable in the sense that no further changes in their ^{31}P-nmr spectra could be detected subsequent to their incubation at 42°C for 120 hr. It thus appears that aging by dealkylation resulted, irrespective of the alkyl residue, in the concomitant appearance of additional ^{31}P-nmr signals 0.25-0.70 ppm upfield relative to the chemical shift signal, at -27.70 ppm, observed and assigned to the aged form, EP-Cht. The new ^{31}P-nmr signals are consistent with the formation of a P-O$^-$ bond, as evidenced from their downfield positioning relative to the non-aged triester OP conjugates. After unfolding in 6 M Gu.HCl, the chemical shifts for all non-aged and aged OP-Cht conjugates moved upfield and became close to the reported values for di- and triester model compounds (see Table 3 and refs. 18 and 24). However, unfolding of the aged form, EP-Cht, obtained by dealkylation, resulted in two distinct ^{31}P-nmr signals (Fig. 9). The lower-field peak corresponded to EP-Cht obtained by use of either EIPF or EP(pNP)$_2$ (i.e., hydrolysis-induced aging). The high-field second signal could be associated with the ^{31}P-nmr chemical shift of either free ethyl alkylphosphoric acid which had been released from a non-covalent association with Cht by treatment with 6 M Gu.HCl, or with a second, discrete OP-Cht conjugate. In order to shed light on the chemical environment of the P atom producing the second ^{31}P-nmr signal observed both in native and denatured OP-Cht conjugates which had been obtained by

dealkylation-induced aging, we carried out the following experiments:

a. The ^{31}P-nmr spectrum of 1.5 mM ethyl pinacolylphosphoric acid (EPinPA) was recorded in the presence of 4 mM native Cht over a period of 72 hr. No change in the chemical shift of the acid was observed relative to that of a sample which had been incubated in the absence of Cht (Fig. 10). Furthermore, it was possible, by dialysis, to completely remove EPinPA from a solution of EPinP-Cht. These results suggest that non-covalent interactions between native Cht, whether free or OP-inhibited, and EPinPA (if, indeed, they occur at all) cannot explain the second ^{31}P-nmr signal observed for aged EP-Cht which had been obtained from EPinP-Cht after incubation at 38^{o}C. Ethyl isopropylphosphoric acid (EIPA) displayed similar results (not shown).

b. As mentioned above, Gu.HCl denaturation of an aged EPinP-Cht conjugate gave rise to two distinct ^{31}P-nmr peaks. When this solution was spiked with fresh EPinPA, the upper-field signal was enhanced significantly (Fig. 11). However, the close proximity of the chemical shift of EPinPA and of the upper field signal of aged EPinP-Cht in 6 M Gu.HCl did not permit a reliable differentiation between the peaks. When an EPinPA-spiked solution of aged EPinP-Cht was dialyzed against 6 M Gu.HCl for 72 hr, the ^{31}P-nmr spectrum became identical with that of the same solution prior to the addition of EPinPA (Fig. 11). In the case of the aged form obtained from EIP-Cht, spiking of an unfolded solution of the OP-Cht conjugate, in 6 M Gu.HCl, with ethyl isopropylphosphoric acid, resulted in the presence of a separate ^{31}P-nmr signal located between the two peaks which are presumed to correspond to two forms of the OP-Cht conjugate. Indeed, extensive dialysis against 6 M Gu.HCl resulted in a ^{31}P-nmr spectrum similar to that observed prior to spiking with EIPA (Fig. 12).

The above results substantiate the assumption that the two peaks which were observed in either native or denatured EP-Cht obtained by heat-enhanced dealkylation are indeed associated with an OP residue which is covalently linked to Cht. Furthermore, the observation that 96 hr of incubation with 0.1 M 3-PAM did not change significantly the ^{31}P-nmr spectra (not shown) of these aged conjugates suggests that both signals represent a P atom environment which is inaccessible to external nucleophiles. One signal appears to correspond to a serine-bound OP moiety which contains the P-O^{-} bond. As for the second signal, we can apparently rule out the possibility of an O->N transfer of a phosphoryl residue since the ^{31}P-nmr spectrum of the aged OP-Cht conjugates did not reveal the presence of a signal corresponding to a phosphoramidate linkage. More experiments will be required in order to fully characterize the second ^{31}P-nmr signal.

3.3. pH-Dependence of the ^{31}P-nmr chemical shift of OP-Cht conjugates

During the ^{31}P-nmr measurements conducted in the course of this project, we also studied the pH-dependence of the chemical shift of both the non-aged (DEP-Cht) and aged (EP-Cht) forms which were obtained by use of paraoxon and EP(pNP)$_2$, respectively. An upfield shift in the ^{31}P-nmr signal with increasing pH (Fig. 13) displayed a titration curve with one titratable group both for DEP-Cht (pK_a = 7.1) and for EP-Cht (pK_a = 10.3). The titration curves for both conjugates were reversible; i.e., the same profile was observed whether titration was carried out from low to high pH or vice versa. Since the titration curves obtained for diethyl- and monoethylphosphoric acid differed from those obtained for the two Cht

conjugates (Fig. 13), it is likely that the difference in pH-chemical shift dependence observed between the two conjugates arises from differences in the interaction of the phosphorus ligand with the polypeptide backbone and not from a difference in dissociation of a P-OH group. It is worth mentioning that EP-Cht obtained by dealkylation of either DEP-Cht or EIP-Cht (not shown) or EPinP-Cht (Fig. 13) displayed the same pH-chemical shift dependence as was observed for EP-Cht which had been prepared by use of $EP(pNP)_2$. These results further substantiate our previous conclusion with regard to the presence of a $P\text{-}O^-$ bond in aged OP-Cht conjugates. During the course of the performance of these experiments, Adebodun and Jordan (47) published similar results which demonstrated, by use of ^{31}P-nmr spectroscopy, an increase of three units in the pK_a of a titratable group of monoisopropylphosphoryl-Cht (pK_a = 10.3) relative to diisopropyl-Cht (pK_a = 7.4).

Changes in proton affinity for the catalytic site residue, His57, of Cht, and of the equivalent His residue in trypsin, were studied earlier by Robillard and Shulman (48) and by Kossiakoff and Spencer (49), who used ^{1}H-nmr spectroscopy and neutron diffraction, respectively. Based on their experimental findings, it was suggested that in the transition state for substrate hydrolysis the pK_a of this histidine in both enzymes is greater than 9.5. Indeed, more recent studies of complexes formed between Cht and either ketone-containing inhibitors (50, 51) or boronic acid derivatives (52), using nmr spectroscopy, have provided supportive evidence for a considerable elevation of the pK_a of a titratable group at or near the active site of Cht relative to the unmodified enzyme. It was consequently suggested that the protonated form of His57 is stabilized by a putative tetrahedral oxyanion adduct which is formed between the active-site serine and the corresponding inhibitors (48-52). Thus, the titration curves presented above, taken together with our X-ray crystallography studies on aged and non-aged OP-Cht conjugates (see below) and with the studies just discussed (48-52), strongly suggest that an electrostatic interaction between a protonated state of His57 and the $P\text{-}O^-$ of the phosphoryl residue of the aged form, EP-Cht, lies at the basis of the resistance of the aged form to reactivation.

3.4. ^{31}P-nmr and reactivation studies at alkaline pH values

In view of the above, it was of interest to study the possibility of displacing a $P\text{-}O^-$-containing phosphoryl residue from an aged OP-Cht conjugate under experimental conditions which may be considered to favor partial dissociation of the $P\text{-}O^-$-imidazolium linkage. We thus investigated the possibility of reactivating EP-Cht by 0.1 M 3-PAM in carbonate buffer at pH 10 (TAble 4). As a control, we first studied the stability of native Cht under these conditions, and the residual activity of a solution of native Cht (ca. 1 μM), at 25°C, was found to be 85 and 40% after 40 and 130 hr, respectively. Increasing the Cht concentration caused significant enhancement of the rate of denaturation/autolysis of the native enzyme; for example, 0.4 mM Cht underwent 70% inactivation during the first 19 hr of incubation at pH 10 in the presence of 0.1 M 3-PAM. When a 1-μM solution of either EIP- or EPinP-Cht was incubated with 0.1 M 3-PAM at either pH 7.8 or pH 10.0, restoration of enzymic activity could be clearly demonstrated over a period of 96-150 hr (Table 4).

Although both the rate and maximum degree of reactivation at pH 10 were lower than at pH 7.8, these results further validate the proposed

reactivation protocol at pH 10. Nevertheless, no recovery of enzymic activity could be detected for EP-Cht subjected to the same protocol, whether at pH 7.8 or at pH 10 (Table 4). It thus appears that partial dissociation of the assumed protonated form of His57 of the aged conjugate, $C_2H_5OP(O)(O^-)$-Cht, is not sufficient to achieve displacement of the P-(O^-)-containing phosphoryl residue from EP-Cht. Indeed, incubation of 1 mM EP-Cht, in the presence of 0.1 M 3-PAM at pH 10.5 (25°C), did not affect the ^{31}P-nmr chemical shift assigned to $C_2H_5OP(O)(O^-)$-Cht. However, upon raising the incubation temperature to 38°C, while maintaining the pH at 10.5, the original ^{31}P-nmr signal disappeared within 48 hr, with concomitant appearance of two peaks at -26.53 and -29.60 ppm, which could be assigned to the diacid $C_2H_5OP(O)(O^-)_2$ and the denatured form of EP-Cht, respectively (Fig. 14). Furthermore, when the same experiment was repeated in the absence of 3-PAM, the ^{31}P-nmr chemical shift of EP-Cht moved upfield to -29.60 ppm, suggesting that almost complete denaturation of EP-Cht had occurred. We note that this transformation was accompanied by the appearance of a low-intensity ^{31}P-nmr signal which corresponded to the above-mentioned diacid, $C_2H_5OP(O)(O^-)_2$. Since the appearance of the diacid ^{31}P-nmr signal was strongly associated with formation of an EP-Cht denatured conjugate, while reactivation was practically undetectable, these observations suggest that displacement of P-(O^-)-containing residues is possible, provided that the three-dimensional environment of the phosphoryl moiety is first perturbed to an extent which renders the phosphoryl residue available to external nucleophiles such as 3-PAM, or even OH^-. In this respect, the denatured (i.e., unfolded) forms of either aged or non-aged OP-Cht conjugates appear to display the same chemical environment for the P atom as is observed in model OP compounds (see Table 3). The results of ^{31}P-nmr chemical shift measurements, taken together with the reactivation data, strongly point to the possibility that the resistance of an aged OP-Cht conjugate to reactivation is due primarily to the interaction of the P-O^- function with His57 and only to a lesser extent to the presence of an electrostatic barrier which prevents the approach of a reactive nucleophile. This conclusion is further supported by the observation that tabun-inhibited Cht, $(CH_3)_2NP(O)(OC_2H_5)$-Cht, also could not be reactivated by 0.1 M 3-PAM. (Table 4). This operationally 'aged' form of OP-Cht does not appear to contain a P-O^- bond, since incubation at pH 11.4 transformed the ^{31}P-nmr signal almost completely into one corresponding to the monoacid, $(CH_3)_2NP(O)(OC_2H_5)(O^-)$ (Fig. 15). It is proposed that locking of the phosphoryl residue within the active-site region at a certain orientation, which renders the inhibited enzyme unreactivatable, indeed involves an interaction between the OP moiety and His57. Irrespective of the nature of this interaction, whether through electrostatic forces, (P-O^-), or through a putative hydrogen bond to the nitrogen of the (P-$N(CH_3)_2$ group, it appears that the magnitude of such a stabilization cannot be estimated from only the observed increase in the apparent pK_a of His57.

4. X-Ray Crystallographic Studies on Aged and Non-Aged OP-Cht Conjugates

X-ray crystallography is, at present, the only way to directly elucidate the three-dimensional structure of a protein. Comparison, by this technique, of homologous pairs of aged and non-aged OP conjugates of Cht and, subsequently, of AChE, would thus yield direct information, at the atomic level, concerning the molecular basis for the aging phenomenon. In the case of AChE, although we now possess a crystalline form suitable for

X-ray studies (53) - which are, in fact, now being carried out in our laboratory - we do not, as yet, have the three-dimensional structure at our disposal. In the case of Cht, however, the three-dimensional structure is well known (19), and has greatly assisted in understanding the mechanism of action of the enzyme. It was shown earlier by Sigler and Skinner (54) that α-Cht in the crystalline state could be almost fully inhibited by DFP, although these authors did not carry out a high-resolution study to elucidate the geometry of the OP-inhibited active site. The advantage of such a procedure, as opposed to crystallization of a preformed OP-enzyme conjugate, is that, if successful, it permits preparation of OP-Cht conjugates isomorphous in crystal structure with the native, unmodified enzyme. Since, as already mentioned, the three-dimensional structure of Cht itself is already known, the structures of the corresponding conjugates can be readily solved. Thus, for example, Ringe et al. (55) have recently adapted this approach for studying the conformational changes induced upon inactivation of Cht by 5-benzyl-6-chloro-2-pyrone; it has not, however, yet been adopted for seeking conformational differences between aged and non-aged OP conjugates of serine hydrolases. Obviously, the same OP's used to produce the homologous pairs of aged and non-aged OP-Cht conjugates used for the spectroscopic studies discussed above, could be similarly used to produce the corresponding crystalline conjugates by soaking Cht crystals in solutions of these OP's under suitable experimental conditions. This study was initiated over 2 years ago, in collaboration with the group of Dr. Joel Sussman in the Dept. of Structural Chemistry at the Weizmann Institute.

The form of Cht selected for these studies was the same one used by Ringe et al. (55), γ-Cht, for which a structure at 1.6 Å resolution was reported by Cohen et al. (56). Crystals of γ-Cht, ca. 2 mm in their long dimension and ca. 1.5 mm across, were grown under crystallization conditions similar to those of Cohen et al. (56), i.e., by precipitation from 50% saturated ammonium sulfate in 0.02 M cacodylate, pH 5.6, at room temperature, for 4 weeks or longer. The non-aged conjugate, diethylphosphoryl-Cht (DEP-Cht), was prepared by dissolving paraoxon in the crystallization medium, at a final concentration of 0.4 mM, and soaking crystals in this solution for up to 4 weeks, with concurrent monitoring of the enzymic activity of sample crystals. It was found that within 4 weeks, >83% inhibition of Cht activity was thus obtained. For further technical details, see Ref. 57

Initial attempts to prepare an aged homolog involved similar incubation of γ-Cht crystals with ethyl bis(p-nitrophenoxy)phosphate ($E(pNP)_2P$). As explained above, this OP should produce an ethyl p-nitrophenoxyphosphoryl conjugate of Cht from which the second pNP group is gradually removed in a 'dynamic' aging process. Unfortunately, the limited solubility of $E(pNP)_2P$ in the crystallization medium, even when predissolved in an organic solvent, prevented straightforward adoption of this approach to obtaining a crystalline aged conjugate, and little or no inhibition of the crystals was observed. This difficulty was subsequently overcome by replacing the ammonium sulfate solution with the organic solvent, 2-methyl-2,4-pentanediol, in which the OP dissolved well. By soaking for 4 weeks, using a 4-mM solution of $E(pNP)_2P$, it was possible to obtain up to 85% inhibition of enzymic activity. However, although the crystals initially retained their original crystal form in the organic solvent, they were rather unstable, eventually deteriorating during the prolonged period needed for soaking in the OP. We subsequently achieved successful preparation of an aged conjugate by use of DFP, acting on the assumption that the

dealkylation reaction involved in aging of this type would occur in the crystalline state, as was, in fact, borne out by the X-ray data (see below). Using 10 mM DFP, in the ammonium sulfate medium, it was possible to inhibit the Cht within the crystal >89% within 10 weeks. Use of DFP yielded the aged conjugate, IP-Cht (see below), which contains one carbon more than the alkyl group(s) in DEP-Cht. In order to produce the exact analog, we made use of another novel compound which we synthesized, namely ethyl isopropylphosphorofluoridate (EIPF). This compound was expected to produce, initially, ethylisopropylphosphoryl-Cht (EIP-Cht), and subsequently, to age quantitatively, with concomitant loss of its isopropyl group, to yield EP-Cht. Exposure of γ-Cht crystals to 10 mM EIPF in the ammonium sulfate medium for 12 weeks produced >95% inhibition of enzymic activity, and the X-ray data (see below) confirmed that aging had indeed occurred to yield the desired structure (i.e., EP-Cht).

Data collection, both for native γ-Cht and for DEP-Cht, was carried out under cryogenic conditions, at 90°K on a Rigaku rotating anode diffractometer, data being collected to resolutions of 1.6 and 1.9 Å, respectively. Refinement of the native γ-Cht started from the 1.9 Å refined room-temperature γ-Cht cooordinates of Cohen et al. (56). The electron density map indicated the presence of a tripeptide, Gly-Ala-Trp (corresponding to the sequence of residues 205-207 in γ-Cht), partially bound in the active site of the native enzyme. This finding is rather similar to the recent report of Dixon and Matthews (58), although these authors identified, under their conditions of data collection and refinement, a somewhat different peptide bound in the active site, which, however, also corresponded to a sequence known to exist in γ-Cht.

The refinement of the DEP-Cht data also started from the room-temperature refined γ-Cht coordinates of Cohen et al. (56). At the initial stages of the refinement of DEP-Cht, the highest positive density peak was found in the active site region. This density fitted the structure of the diethylphosphate part in the crystal structure of silver diethylphosphate determined by Hazel and Collin (59). In the refined structure, the diethylphosphoryl group is covalently bound to Ser195 O^{γ}, with a bond length of 1.55 Å to the P atom. The structure of the crystalline 'aged' conjugate, IP-Cht, was similarly refined from a 2.1 Å resolution data set, collected at room temperature, on a Siemens/Nicolet area detector. At the initial stages of refinement of the 'aged' conjugate, the highest positive density peak was located in the active-site region. The density clearly showed that the diisopropylphosphoryl moiety had lost one of its isopropyl groups, leaving a monoisopropyl (IP) group bound to the enzyme (IP-Cht). The density was fitted by using the structure of the DIP part of the crystal structure of diisopropyl-(2,3,4,5-tetraphenyl-cyclopenta-1,4-dienyl)phosphate determined by Krishnanachari and Jacobson (60). In this 'aged' conjugate, IP-Cht, the OP group is covalently attached to Ser195 O^{γ}, with a bond length of 1.54 Å to the P atom. Data collection for the second crystalline 'aged' conjugate, EP-Cht, was carried out under the same experimental conditions as for native Cht and for DEP-Cht, to a resolution of 1.9 Å. Partial refinement yielded a map which appeared to superimpose with that obtained for IP-Cht. Because of this and in view of the substantial cost of the computer time involved, further refinement was thus deemed superfluous

The overall structures of the aged and non-aged conjugates superimpose very well, as can be seen by from inspection of Fig. 16. A more detailed

picture of the situation prevailing within the active-site is given in Figs. 17 and 18 and by inspection of the interatomic distances summarized in Table 5.

Table 5 : Interatomic Distances (A) Among Selected Residues in the Active Site Region of Aged and Non-Aged OP-Cht Conjugates.

Atom 1	Atom 2	(isoPrO)P(O)(O^-)-Cht	$(C_2H_5O)_2P(O)$-Cht
Ser195 O^{τ}	His57 $N^{\epsilon 2}$	3.09	3.16
His57 $N^{\epsilon 2}$	O2	2.60	3.58
Ser195 N	O4	3.18	2.93
Gly193 N	O4	2.94	2.83
Asp102 $O^{\delta 2}$	His57 $N^{\delta 1}$	2.79	2.83
Asp102 $O^{\delta 1}$	His57 N	2.82	2.86
Asp102 $O^{\delta 1}$	Ala56 N	2.99	2.71
P1	O4	1.44	1.44
P1	O2	1.54	1.54
P1	O3	1.54	1.52
P1	Ser195 O^{τ}	1.54	1.55

for diagramatic scheme showing the specified atoms see Fig.

The interatomic distances clearly show, as expected, that in both conjugates, the phosphoryl group is covalently attached to the enzyme through the oxygen atom labelled Ser195 O^{γ}. The phosphoryl oxygen atom (P=O), designated O4, of both conjugates forms two hydrogen bonds with the amide nitrogen atoms labelled Ser195 N and Ser193 N. Thus, in both conjugates O4 is directed towards the region of the enzyme known as the 'oxyanion hole' (61). The most striking difference between the two conjugates is the movement of the oxygen designated as O2, which is negatively charged in the aged conjugate, towards the imidazole nitrogen labelled His57 $N^{\epsilon 2}$. The short distance between the two atoms (2.60 Å) indicates possible existence of a salt-bridge between the P-O^- and the positively charged imidazole ring. In contrast, the corresponding O2 atom in the non-aged conjugate is 3.58 Å distant from His57 $N^{\epsilon 2}$; thus, the C_2H_5O-P linkage appears to be more amenable to free rotation within the active-site pocket than its O^--P counterpart. Furthermore, visual inspection does not reveal any close interaction (<4.0 Å) of this ethoxy group with the protein backbone. In contrast, the other ethoxy group, corresponding to O3, appears close to C190 and C215; even more striking is the proximity of the homologous group, the isopropoxy moiety in the aged conjugate, to $C^{\alpha}191$, C191 and $C^{\alpha}215$. These residues have previously been assigned as making up part of the hydrophobic pocket (62 and references therein). The refined structures of the aged and non-aged OP-Cht conjugates further reveal that the relative positioning of the active-site residues, Ala56, His57, Asp102, Ser195

and Gly193, is maintained practically unchanged in the two conjugates, as judged by the criterion of interatomic distances (Table 5) and torsion angles (Table 6). Particularly striking is the finding that the distance Ser195 O^{γ}....His57 $N^{\varepsilon 2}$ is 3.09 Å and 3.16 Å in the aged and non-aged conjugates, respectively, and is 2.99 Å in the native enzyme. Visual inspection of the active-site region of both conjugates indicates that the bound OP residue is accessible to the approach from without of a small molecule (e.g. an oxime) to the same degree.

The crystallographic data presented above are in excellent agreement with the physicochemical data obtained in solution, by optical and ^{31}P-nmr spectroscopy, inasmuch as they provide direct evidence for strong electrostatic interaction of the negatively charged OP moiety in the aged OP-Cht conjugate with the imidazolium group of His57. Since the X-ray data did not present any evidencefor gross conformational differences which might help to explain the resistance of the aged conjugate to reactivation, it remains to be established how this interaction results in the resistance to nucleophilic reactivation (see below).

Table 6 : Torsion Angles (^{o}) of Selected Residues in Aged and Non-Aged OP-Cht conjugates.

Bond	(isoPrO)P(O)(O^-)-Cht	$(C_2H_5O)_2P(O)$-Cht
Ser195 X1	-71	-72
His57 X1	84	89
His57 X2	-93	-98
Asp102 X1	-167	-173
Asp102 X2	-163	-162

5. Comparative Evaluation of the Significance of the Results Obtained by the Diverse Experimental Techniques Utilized in the Framework of the Project

Our initial efforts to provide a rationale, at the molecular level, for the resistance to reactivation of aged OP conjugates of serine hydrolases focused on the use of fluorescence (16, 17) and nmr (18) spectroscopy. In the framework of the present project, we further extended our experimental repertoire by use of the techniques of circular dichroism and transverse urea gradient electrophoresis, to which we have added the powerful technique of X-ray crystallography. The diversity of experimental techniques

employed generated different types of data which could be comparatively analyzed in our efforts to understand the chemical nature of the aging process.

The techniques of circular dichroism and transverse urea gradient electrophoresis were both utilized to evaluate the possibility that the aging process was accompanied by a concomitant stabilization of the OP-enzyme conjugate, as discussed above, the two techniques yielded similar information, which indicated that for two conjugates of AChE, aging is accompanied by a substantial stabilization, which can be estimated to be of the order of a few kilocalories per mole of enzyme. Such a value is similar to values associated with single noncovalent interactions in other experimental systems (40, 41) and could, therefore, be attributed to a putative interaction with a nearby positive charge of the negative charge generated by aging. This finding is in excellent agreement with our most recent ^{31}P-nmr observations: Thus we were able to show not only that aging involves dealkylation of the enzyme-bound OP group, but also that the negative charge so generated elevated the pK_a of a single titratable residue, presumably $N^{\varepsilon 2}$ of His57, to an extent consistent with the energy stabilization observed in the denaturation experiments discussed above. The X-ray crystallography data allowed us to observe directly the structural basis for these observations, namely the movement of the negatively charged oxygen atom of the phosphoryl group of the aged conjugate towards $N^{\varepsilon 2}$ of His57. The three experimental approaches thus yielded a unified interpretation of the chemical changes which lie at the basis of the aging phenomenon, at least in the case of Cht. It is worth noting that the CD and transverse urea gradient electrophoresis data gave additional information. Thus, even though the X-ray crystallographic measurements showed that no change in the overall conformation of the OP-Cht conjugate occurred upon aging, interaction of the $P\text{-}O^-$ group with His57 resulted in an overall stabilization of the whole protein molecule, whose cooperative unfolding was retarded upon aging.

The above conclusions are somewhat in conflict with the interpretation of our earlier experiments in which we employed pyrene-containing aged and non-aged organophosphoryl conjugates of AChE (16) and Cht (17) to demonstrate significant conformational differences between the aged and non-aged conjugates. Particularly striking is the marked difference in the CD spectra, in the pyrene region, of the appropriate aged and non-aged OP-Cht conjugates, when considered in relation to the X-ray data on DEP-Cht and IP- (and also EP-) Cht. Although we do not, unfortunately, have X-ray data for the pyrene-containing conjugates, there is nothing in the available X-ray data to indicate what might cause the major change in CD spectrum observed. Thus, in the three-dimensional structures yielded by the X-ray data, the residual alkyl chain (isopropyl) in the aged conjugate lies pretty much along the same axis as in the non-aged conjugate (ethyl). Therefore, assuming that the pyrene moiety is oriented towards the hydrophobic pocket mentioned earlier, it is difficult to see how the pyrene group, which lies at the end of a four carbon spacer, could be pulled, by aging, into a different orientation which could explain the CD spectra observed. It should be remembered that, although our kinetic data for inhibition of Cht by PBEPF clearly show strong noncovalent binding (18), most likely with the hydrophobic patch in the substrate-binding pocket, we do not have any direct evidence that the pyrene group in the aged and non-aged conjugates occupies the same locus. It is known, from other experimental systems, that quite small atomic movements in one part of a protein

molecule may generate surprisingly large movements in another region, e.g., on binding of oxygen to hemoglobin (see Figs. 8-22 and 8-23 in Ref. 63) Thus, even the small movement observed in the position of the phosphorus atom might have an amplified effect on the positioning and/or orientation of the pyrene ring. Only X-ray measurements on crystalline derivatives of the conjugates themselves could definitively settle this issue. It should, of course, also be kept in mind that structures in the crystalline state may not faithfully reflect the corresponding structures in solution.

One conclusion from our multidisciplinary research effort is that although fluorescent probes can serve to indicate the presence of differences such as those which exist between aged and non-aged OP conjugates of serine hydrolases, any definitive interpretation necessitates the use of X-ray crystallography. In this context it should be pointed out that the volume of spectroscopic data generated thus far for the pyrene-containing OP ligands would justify an effort to study the three-dimensional structure of crystalline preparations of the corresponding aged and non-aged conjugates. It is envisaged that the results so obtained, taken together with complementary data obtained in solution, would enable us to 'fine-tune' the design of additional fluorescent probes, including those of the 2-naphthol family which, due to their proton donor properties, are ideally suited to studying the aging problem.

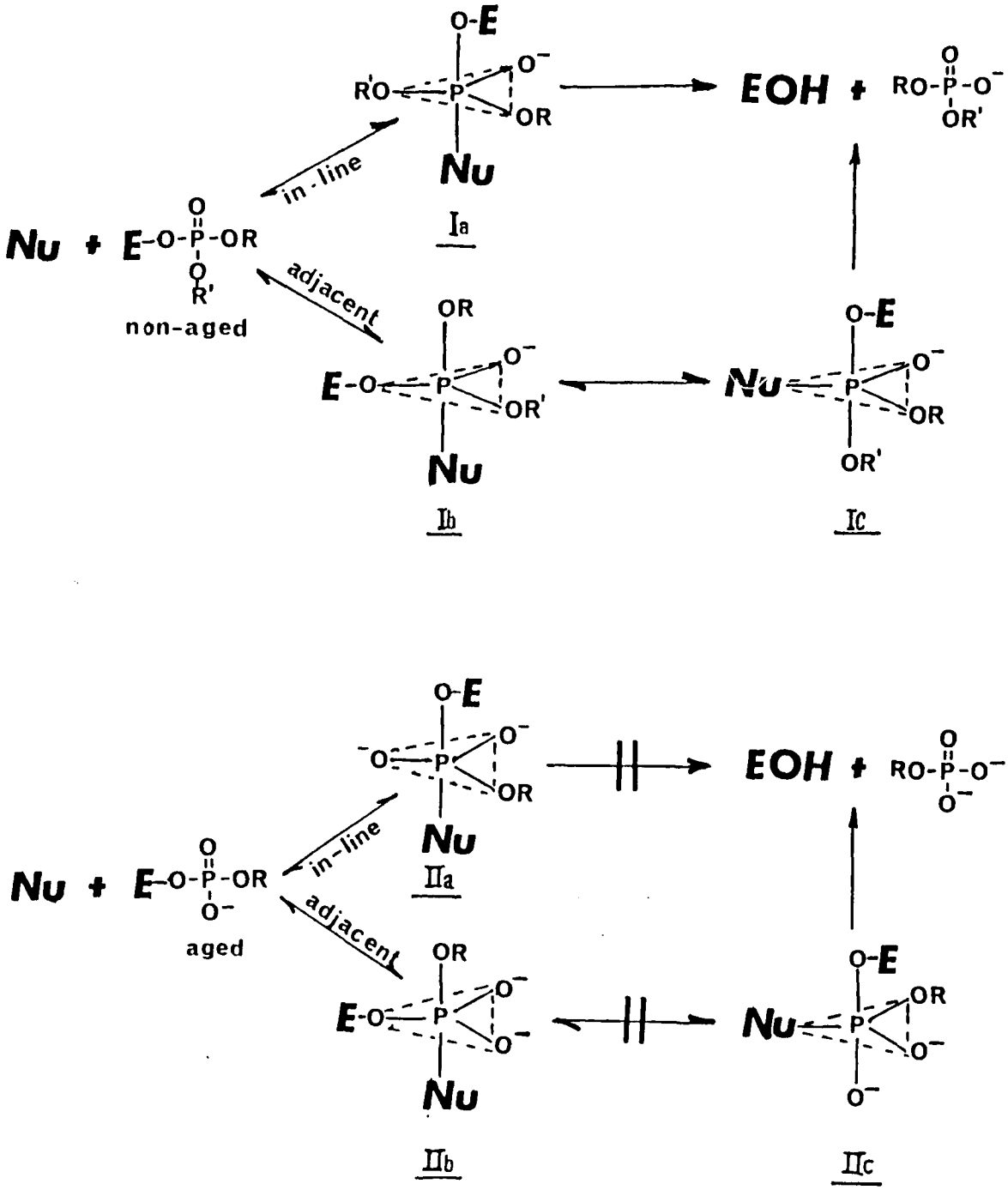
6. Possible Explanations for Aging-Induced Resistance to Reactivation

The X-ray data presented strongly indicate that conformational changes of the polypeptide backbone in or around the active site of γ-Cht do not, in general, lie at the basis of the unusual resistance of the aged conjugates to reactivation by nucleophiles such as 3-PAM. Other possibilities must, therefore, be considered. The fact that neither $(C_2H_5O)P(O)(O^-)$-Cht nor $(C_2H_5O)P(O)(N(CH_3)_2)$-Cht could be reactivated argues against a major role for simple electrostatic repulsion in the resistance to reactivation which results from aging. Since the phosphoryl moieties in the two above-mentioned conjugates can be displaced following denaturation of the corresponding conjugates, the most likely conclusion is that a unique interaction between the $P\text{-}O^-$ group (and possibly also the $P\text{-}N(CH_3)_2$ group) and the positively charged imidazole of His57 imposes a severe restraint on the reactivation process. Apparently displacement of the phosphoryl moiety in the aged conjugate cannot occur by the same route as taken by the non-aged conjugate. Although the finer details of the phosphorylation and dephosphorylation of serine hydrolases have not been fully worked out, we will adopt the pathway depicted in Scheme 11 as the framework for our consideration of possible factors involved in the resistance of aged OP-Cht conjugates to reactivation.

It is widely accepted that displacement reactions at tetrahedral phosphorus atoms proceed through a penta-coordinated species with a trigonal bipyramidal geometry (TGP) (64, 65). The TGP structure has two apical (axial) positions, which are usually considered to be occupied by the attacking nucleophile and by the leaving group in the initial complex. The remaining three ligands are bound at equatorial positions which form the basal plane of the TGP. Assuming that similar mechanisms hold for the experimental system under consideration, reactivation of the non-aged conjugate may occur via an in-line attack (Ia) which places the leaving group

(i.e., the enzyme) in an apical position in the initial penta-coordinate intermediate, as has been suggested to occur in several enzyme-catalyzed phosphoryl transfer reactions (65). It is possible, however, that the enzyme is located at an equatorial position in the penta-coordinate complex formed initially (adjacent displacement, see Ib and IIb as opposed to Ia and IIa). In such a case, a putative ligand rearrangement, known as Berry pseudorotation (66), could bring the enzyme into an apical position which would permit departure and, thereby, decomposition of the penta-coordinate TGP intermediate. Although a preference of Cht for either of the two positions is not known, it is possible that, in contrast to the non-aged conjugate, the 'aged' enzyme is forced into an equatorial position (adjacent displacement) along with the two $P\text{-}O^-$ bonds (IIb). If this is indeed the case, it may be argued that pseudorotation (IIb -- IIc), to bring the enzyme into an apical position, will not occur readily because, according to the preference rules, the $P\text{-}O^-$ bond must occupy an equatorial site (67). In the case of the non-aged conjugate, if the enzyme is oriented equatorially, pseudorotation is formally permitted (Ib -- Ic), since the single $P\text{-}O^-$ bond serves as a pivot for the pseudorotation process while maintaining its equatorial position. The observation that the phosphoryl residue can be displaced from the denatured form of the aged conjugate suggests that an in-line mechanism is permitted subsequent to denaturation even though, formally, the denatured and native species correspond to the same diester-monoanion organophosphate. We propose, therefore, that the strong interaction between $N^{\varepsilon 2}$ of His57 and either $P\text{-}O^-$ or $P\text{-}N(CH_3)_2$ in the native conjugate forces the enzyme into an equatorial position; since, as already discussed, pseudorotation may be restricted under these circumstances, detectable reactivation will, thereby, be prevented. Although the above discussion focused on the possible formation of a TGP with both apical entry and departure, other mechanisms cannot be ruled out. These may include various combinations of apical and equatorial entry and departure, even though such alternate mechanisms are only rarely encountered in the literature (68). An alternative explanation for the resistance to reactivation of the aged conjugate, which might also be considered within the framework of the pathways illustrated in Scheme 11, would involve, as a rate-limiting step, proton transfer to the leaving group, i.e., the enzyme, from or with the mediation of the $N^{\varepsilon 2}$ of His57. Indeed, measurements of solvent isotope effects and of proton inventories suggest that proton transfer may be involved in the rate-limiting step of substrate hydrolysis by serine hydrolases. The pK_a of His57 has been demonstrated by ourselves and by others (45) to increase from ca. pH 7 to pH >9.5 when the non-aged conjugate is transformed to its aged counterpart. It is envisaged, in the case of the aged conjugate, that formation of the penta-coordinate intermediate would introduce a second $P\text{-}O^-$ bond (IIa or IIb) which might drive the basicity (i.e., the pK_a) of His57 to even higher values. If, indeed, $N^{\varepsilon 2}$ of His57 participates in dephosphorylation, proton transfer to the leaving group would thus be affected dramatically.

It should be pointed out that we do not have direct evidence to support the notion that steric restraints introduced by the $P\text{-}O^-$ bond prevent the Ser195 $O\gamma$ from occupying the apical position. In contrast, we do have experimental evidence which is consistent with the alternative explanation presented above. Obviously, both these factors could contribute to the unusual resistance observed experimentally.



SCHEME 11

Proposed penta-coordinated species with trigonal-bipyramidal geometry as possible intermediates in displacement reactions of OP-Cht conjugates (E, Cht; Nu, nucleophile such as 3-PAM)

7. P-N-Containing OP Conjugates of Serine Hydrolases

As detailed above, in our preliminary experiments involving characterization of the novel 2-naphthol-containing OP's which we synthesized, it was observed that conjugates both with AChE and BChE were rather resistant to reactivation by 2-PAM, even though fluorescence measurements clearly showed retention of the 2-naphthol group in the conjugates. Since our experimental difficulties had driven us to preparation of P-N type, rather than P-O-type, naphthol ligands, it occurred to us that the observed resistance might lie in the chemical nature of the ligand. This was because it has been reported that inactivation of AChE by P-N OP's, such as tabun, results in conjugates which are difficult to reactivate (37, 38). Accordingly, we prepared the tabun conjugate of Cht, and were able to clearly demonstrate, using ^{31}P-nmr spectroscopy, that resistance to reactivation, in this case, was not accompanied by detectable detachment of the substituents of the phosphorus atom.

These observations led us to seek a common denominator between conjugates of P-N-containing OP's and 'aged' conjugates of P-O-containing OP's, the latter of which had been shown to contain a $P\text{-}O^-$ function. Taking into account the considerations outlined above with respect to the mechanistic basis of 'aging', it seemed to us that an attractive explanation for the analogous phenomenon in the case of the P-N-containing OP's might be the formation of a bond between the free electron pair of the nitrogen atom of the OP residue and the protonated form of $N^{\varepsilon 2}$ of His-57. Although we are, of course, aware that protonation of the OP nitrogen should offer a route to cleavage of the P-N bond (69, 70), it possible that the same stereochemical constraints discussed above with respect to nucleophilic displacement may also be valid in this case. A direct test of this possibility would be offered by solution of the three-dimensional structure of the tabun conjugate of Cht by X-ray crystallography.

SUMMARY AND CONCLUSIONS

1. Novel naphthol-containing OP's were synthesized and characterized with respect to their ability to serve as AChE inhibitors. Although they react weakly, they do produce OP-AChE conjugates in which the naphthol group is retained. In view of the spectral properties of the chromophore, it is envisaged that the novel probes will prove valuable in future studies of the mechanisms of inhibition of AChE by OP's and of reactivation of the conjugates; this with the proviso that present problems with respect to stoichiometry can be overcome.

2. Our observation that the naphthol-containing OP's of the P-N type produced conjugates, both with AChE and BChE, which could not be reactivated, suggested a resistance to reactivation which is not due to dealkylation, since the fluorophore was retained in the resistant conjugate. This supposition was directly substantiated by ^{31}P-nmr studies on tabun-Cht conjugates. These findings provide evidence that dealkylation, with concomitant production of a P-O$^-$ bond, is not a prerequisite for 'aging'. This, in turn, suggests the further use of P-N type OP's as tools for understanding the molecular basis of the aging reaction.

3. The relative stability of homologous pairs of aged and non-aged OP conjugates of Cht and AChE was investigated. The two techniques employed (monitoring of the CD spectrum of the conjugates as a function of increasing guanidine hydrochloride concentration and transverse urea gradient electrophoresis) revealed that the aged conjugates are substantially more resistant to denaturation than the non-aged conjugates and that the energies involved are consistent with the formation of a single salt-bridge.

4. ^{31}P-nmr studies employing various OP-Cht conjugates demonstrated that aging via either hydrolysis or dealkylation both lead to the same P-O$^-$-containing end product. In contrast to the aged products obtained by hydrolysis at room temperature, the products of thermally accelerated dealkylation displayed additional ^{31}P-nmr signals which are consistent with the formation of a second P-O$^-$-containing species. This second species does not appear to arise from an intramolecular alkylation reaction, since: a) ^{1}H-nmr data clearly demonstrate the absence of a protein-bound pinacolyl moiety in the thermally aged OP conjugate of Cht obtained by use of ethyl pinacolylphosphorofluoridate (data obtained subsequent to the termination of this grant); b) X-ray crystallography of the crystalline aged Cht conjugate (MIP-Cht) obtained by use of DFP does not reveal a second isopropyl group attached in the vicinity of the active site. The increase, by three orders of magnitude, in the basicity of the catalytic histidine, His57, is consistent with the formation of a salt-bridge between the negatively charged oxygen produced by aging and the imidazolium ring.

5. X-ray crystallography showed that the overall conformation of an aged OP-Cht conjugate was very similar to that of a non-aged counterpart. The only significant difference detected arose from a reorientation of the bound OP moiety due to a movement of the negatively charge oxygen atom produced by aging towards the imidazolium ring of His57 with presumptive formation of a salt bridge.

6. The various experimental techniques employed all point to the formation of a salt-bridge between the negatively charged oxygen atom produced by

aging and the imidazolium group within the catalytic site of Cht (and presumably also of cholinesterases). This clearly explains the increased conformational stability of the aged enzyme. It does not, however, offer an explanation for the unusual resistance to nucleophilic reactivation. Displacement reactions at phosphorus atoms are believed to proceed by a pentacoordinate intermediate. It is possible that the salt-bridge produced by aging does not permit the substituents around the P atom to assume the geometry required in order for displacement to occur. Alternatively, the enhanced basicity of the imidazole observed in the ground state may be further increased in the transition state; as a consequence, proton transfer from the imidazolium ring to the leaving group might be retarded to an extent which would preclude experimentally detectable reactivation. A combination of these two restrictions should also be considered.

7. The above conclusions suggest a number of experimental approaches which might prove fruitful in clarifying the molecular events underlying the aging process. These may be briefly summarized as follows:

a. ^{31}P-nmr studies of enzyme conjugates of P-N-type OP's.

b. Studies of the reactivation of aged conjugates at elevated pH values, employing enzymes whose OP conjugates are known to be more amenable to reactivation than the corresponding OP-Cht conjugates (e.g. AChE and BChE).

c. Use of suitable tailor-made OP ligands to investigate the apicophilicity preference of serine hydrolase during phosphorylation and dephosphorylation.

d. Measurement of the effect of D_2O on the phosphorylation and dephosphorylation of serine hydrolases.

e. X-ray crystallographic studies of tabun-inhibited Cht and of pyrene-containing OP-Cht conjugates.

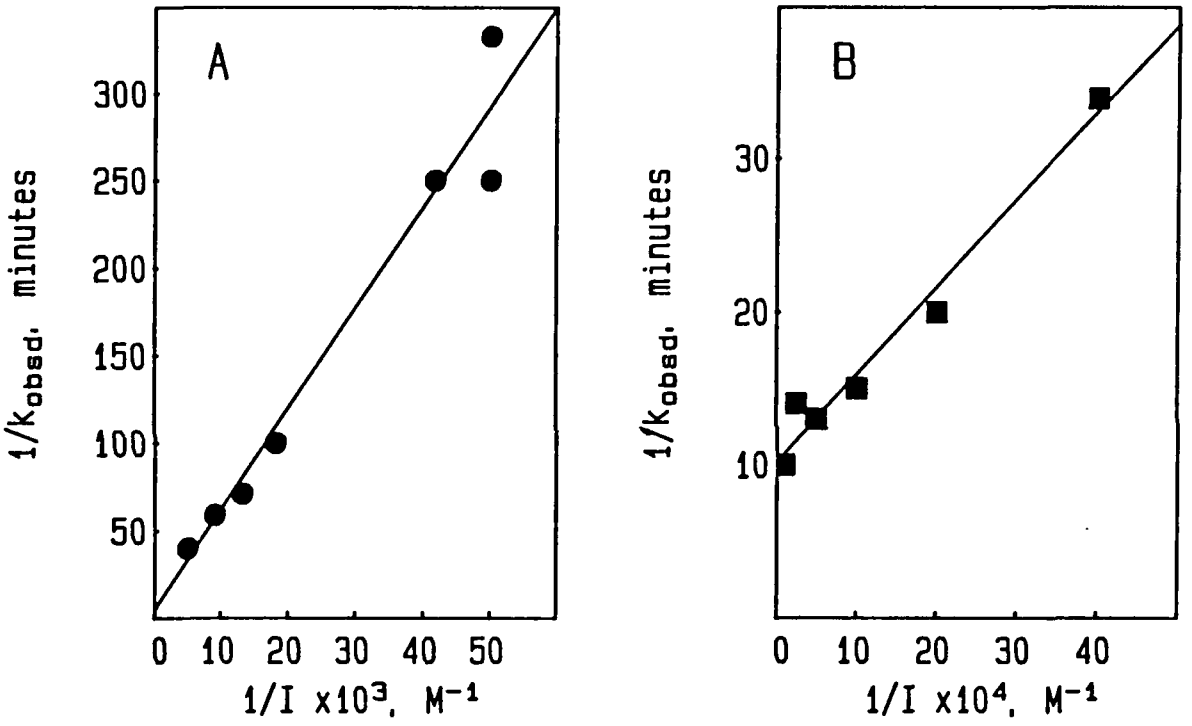


Fig. 1: Double reciprocal plots for the inhibition of Torpedo AChE and human serum BChE by EP(pNP)NPA. A) AChE; B) BChE. Inhibition was performed in 0.05 M phosphate buffer, pH 8.0, containing 1% acetonitrile, at 25-26°C. Rate constants were calculated in accordance with the equation: $1/k_{obs} = (K_I/k')(1/I) + 1/k'$

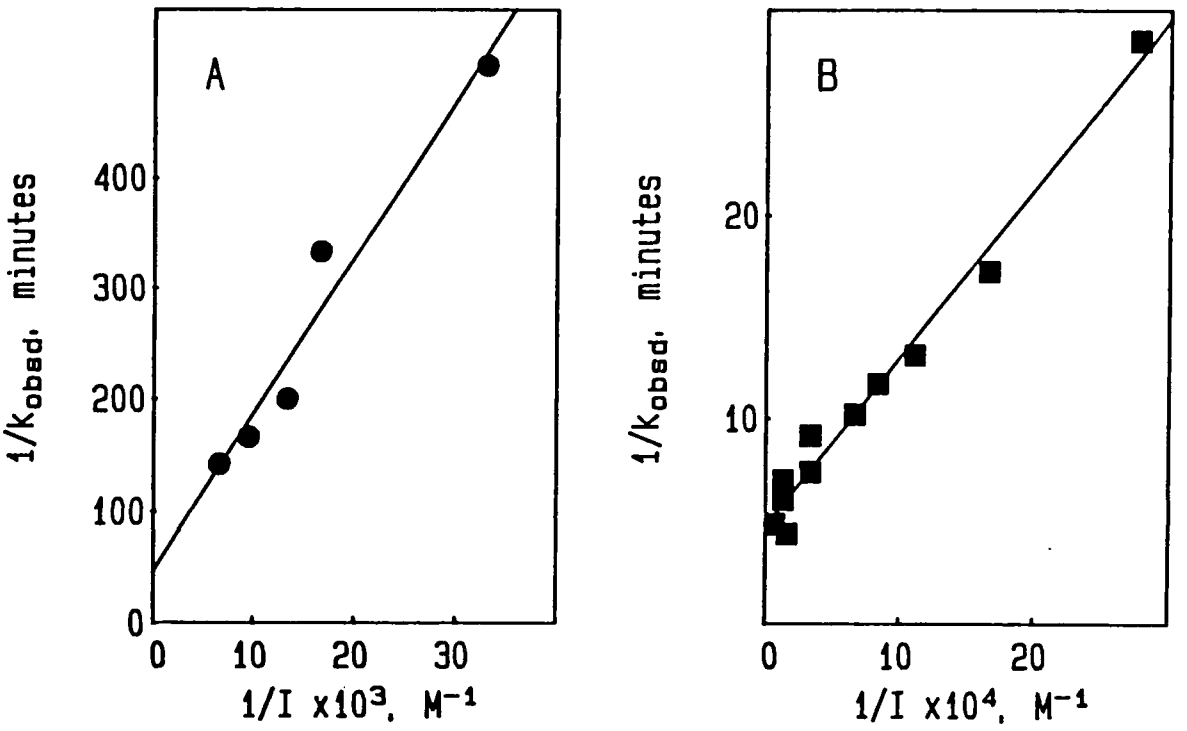


Fig. 2: Double reciprocal plots for the inhibition of Torpedo AChE and human serum BChE by IP(pNP)NPA. A) AChE; B) BChE. Experimental details and plotting of data were performed as in Fig. 1.

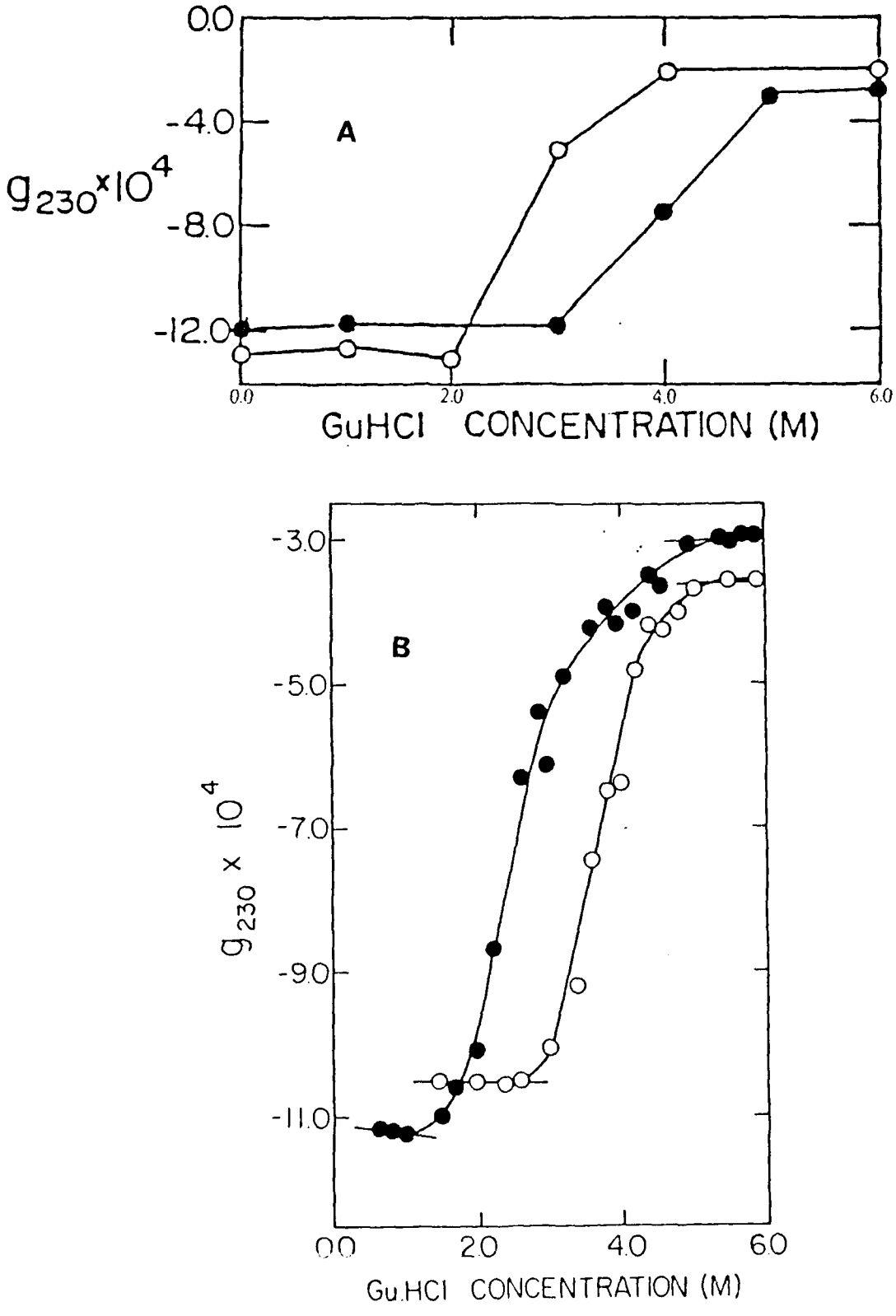


Fig. 3: Dependence of the absorption anisotropy factor at 230 nm, g_{230}, on the guanidine hydrochloride concentration for aged and non-aged OP conjugates of Cht. A) DEP-Cht, O - O, and EP-Cht, ● - ●; B) PBEP-Cht, ● - ●, and PBP-Cht, O -O .

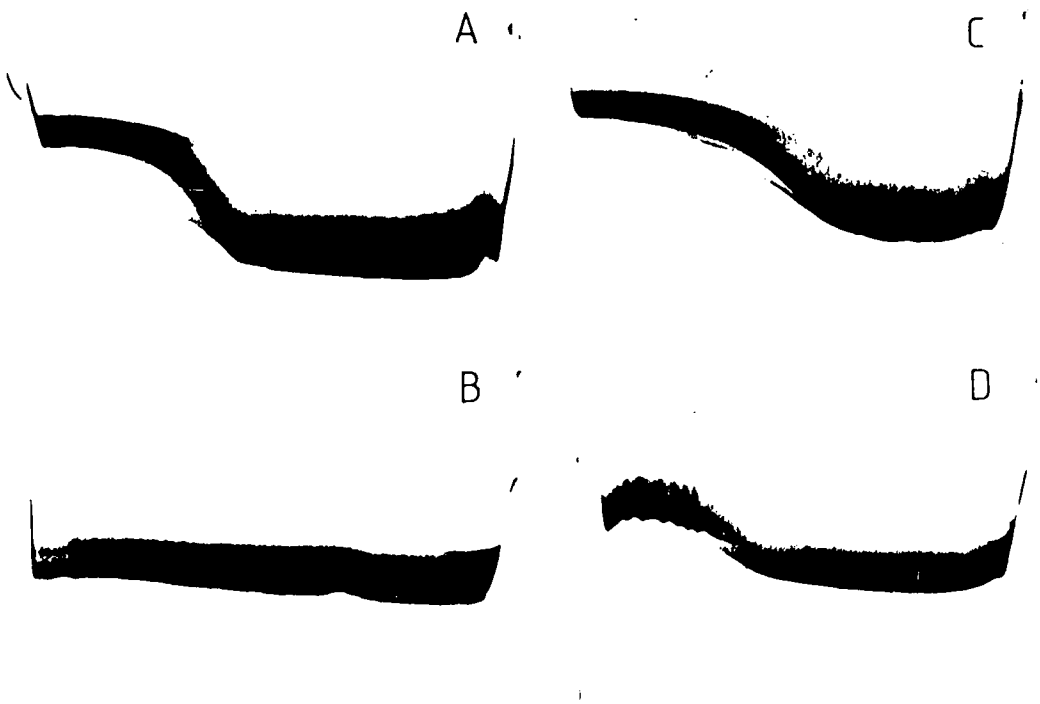


Fig. 4: Transverse-urea-gradient electrophoretograms of DEP-Cht (A, C) and EP-Cht (B, D) run at 25°C (A, B) and 37°C (C, D). The urea concentration within the gels increases linearly from 0 to 8 M from right to left.

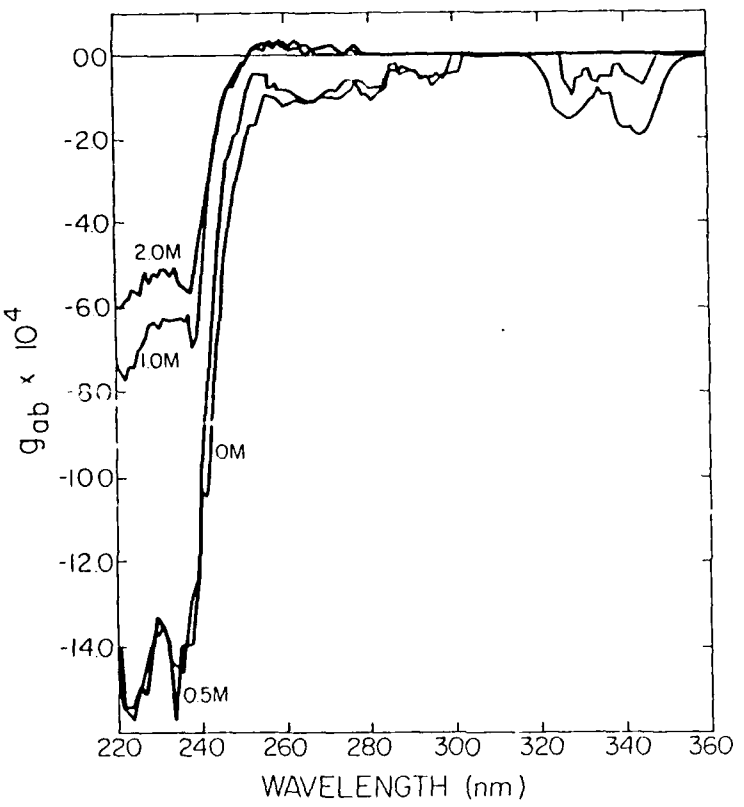


Fig. 5: Circular dichroism spectrum of PBEP-AChE as a function of the guanidine hydrochloride concentration. The concentration of the conjugate was 1×10^{-5} M active sites.

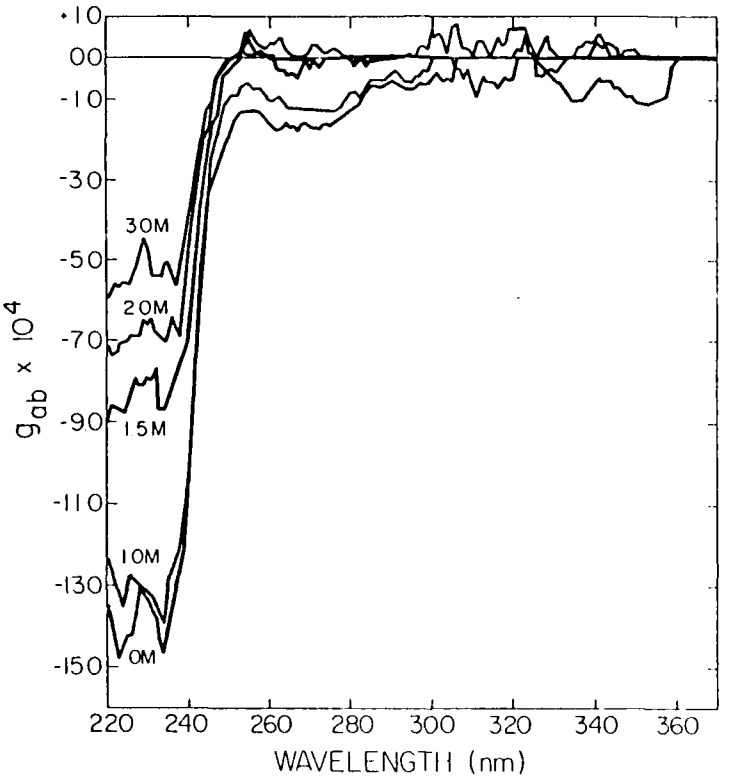


Fig. 6: Circular dichroism spectrum of PBP-AChE as a function of the guanidine hydrochloride concentration. The concentration of the conjugate was $1x10^{-5}$ M active sites.

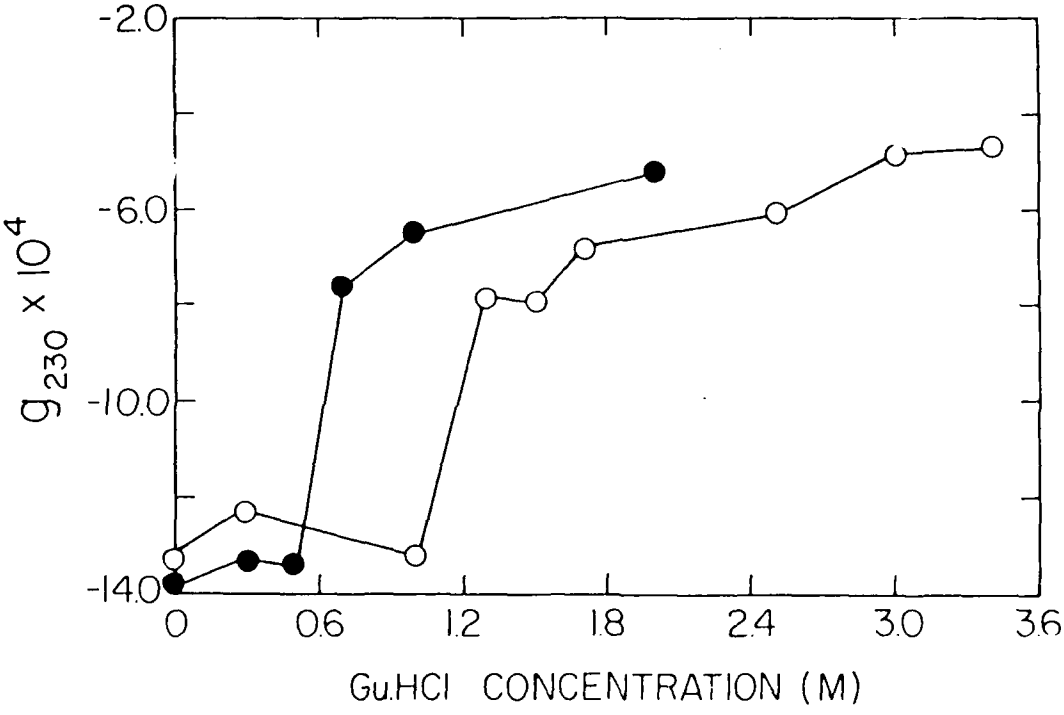


Fig. 7: Dependence of the absorption anisotropy factor at 230 nm, g_{230}, on the guanidine hydrochloride concentration for aged and non-aged OP conjugates of AChE. PBEP-AChE, ● - ●, and PBP-AChE, O - O.

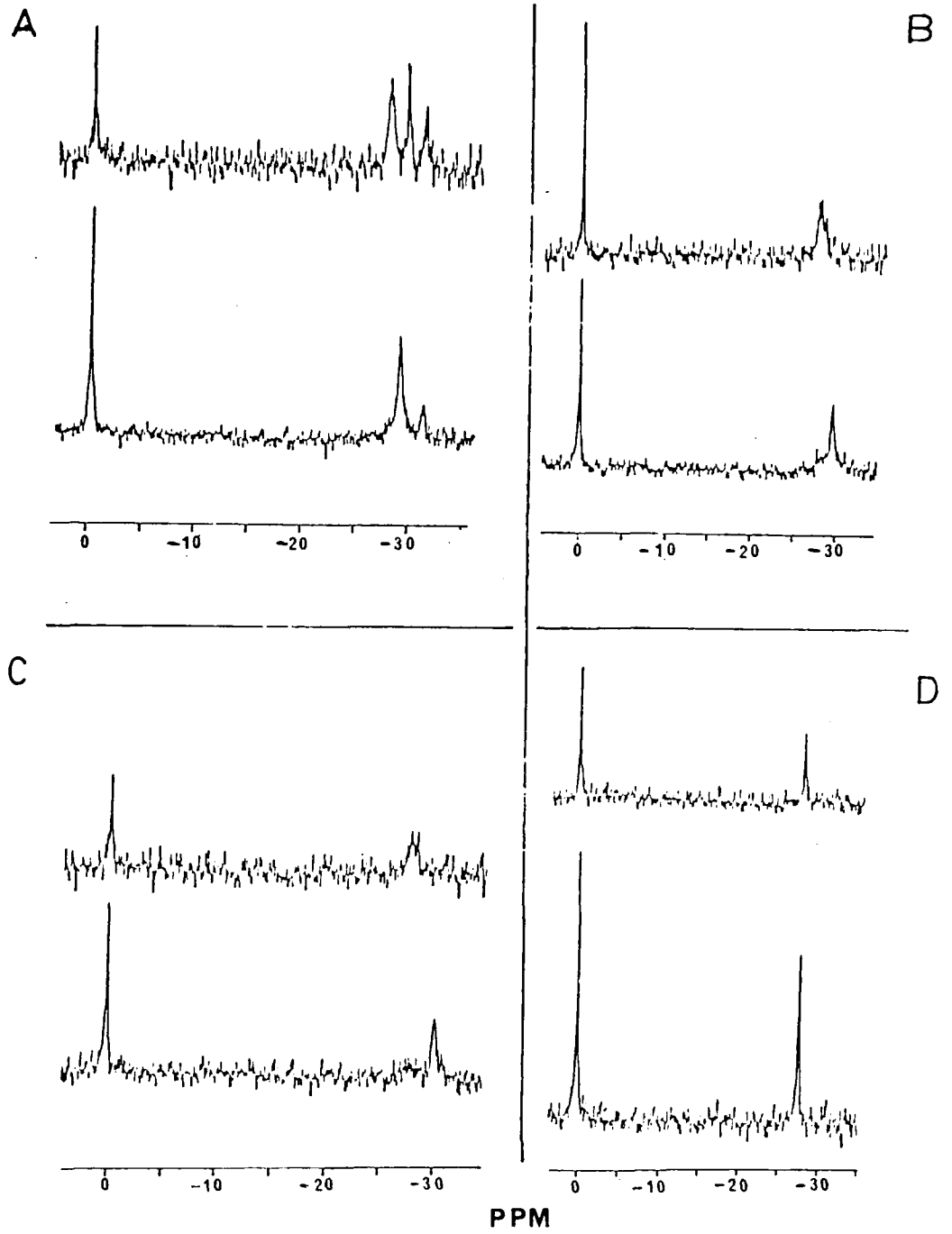


Fig. 8: ^{31}P-nmr spectra of OP-Cht conjugates showing conversion of non-aged to aged conjugates upon heat treatment. Heating was carried out in 0.1 M Tris chloride, pH 7.0, using 0.8-2 mM solutions of the conjugates. In each panel, the lower and upper lines present spectra of control and heat-treated (38^{o}C, 120 h) samples, respectively, the spectra being taken following extensive dialysis. Negative signs indicate an upfield chemical shift relative to HMPA (ppm). A) $(C_2H_5O)_2P(O)$-Cht; B) $C_2H_5OP(O)(OisoPr)$-Cht; C) $C_2H_5OP(O)(OPinacolyl)$-Cht; D) $C_2H_5OP(O)(O^-)$-Cht.

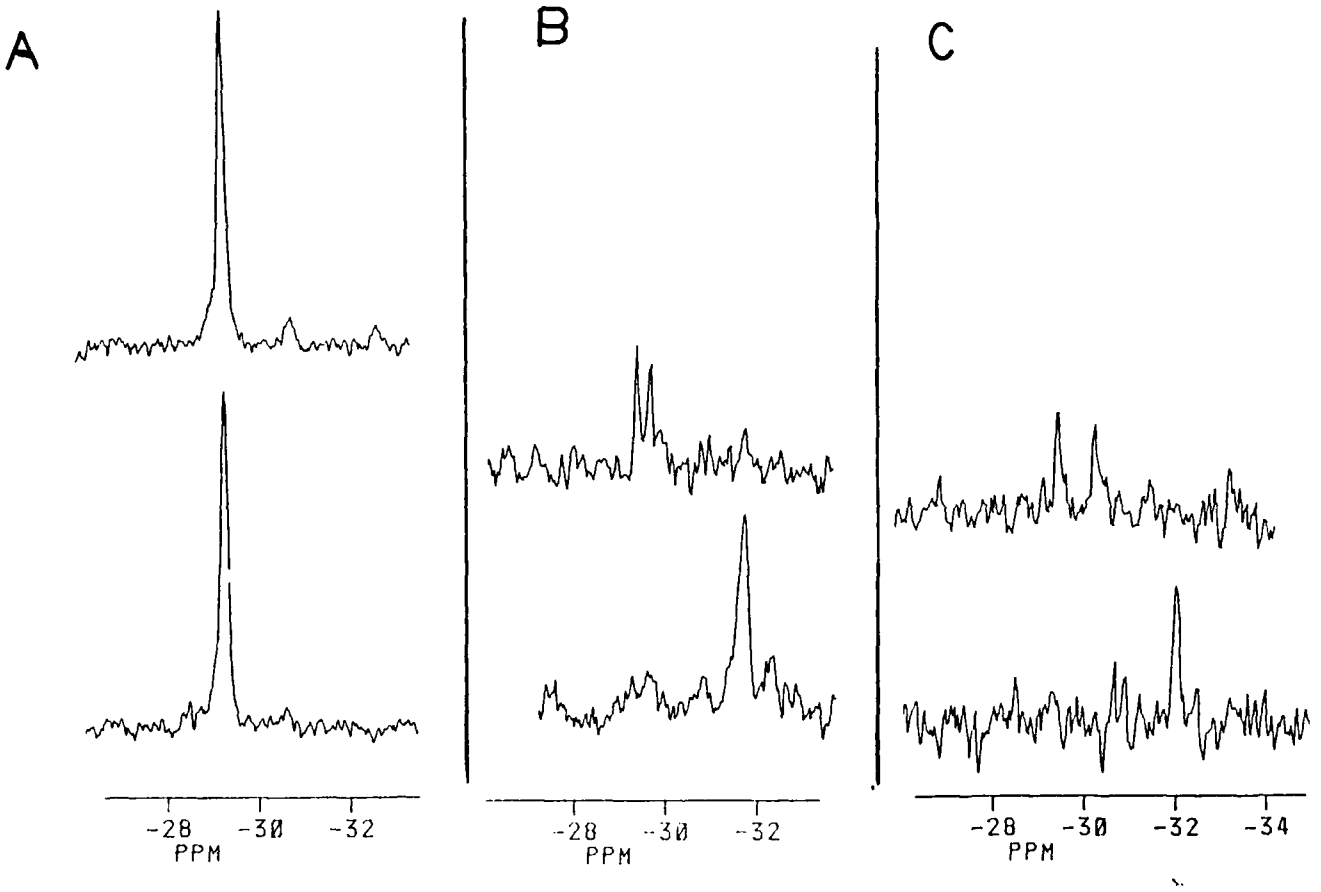


Fig. 9: ^{31}P-nmr spectra of OP-Cht conjugates in 6 M Gu.HCl. Measurements were carried out at pH 7.0 (0.1 M Tris chloride) using 0.8-2 mM solutions of the conjugates. In each panel, the lower and upper traces represent spectra of fresh and heat-treated (see Fig. 8) samples, respectively. Negative signs indicate an upfield shift relative to HMPA. A) $C_2H_5OP(O)(O^-)$-Cht; B) $C_2H_5OP(O)(OPin)$-Cht; C) $C_2H_5OP(O)(OisoPr)$-Cht.

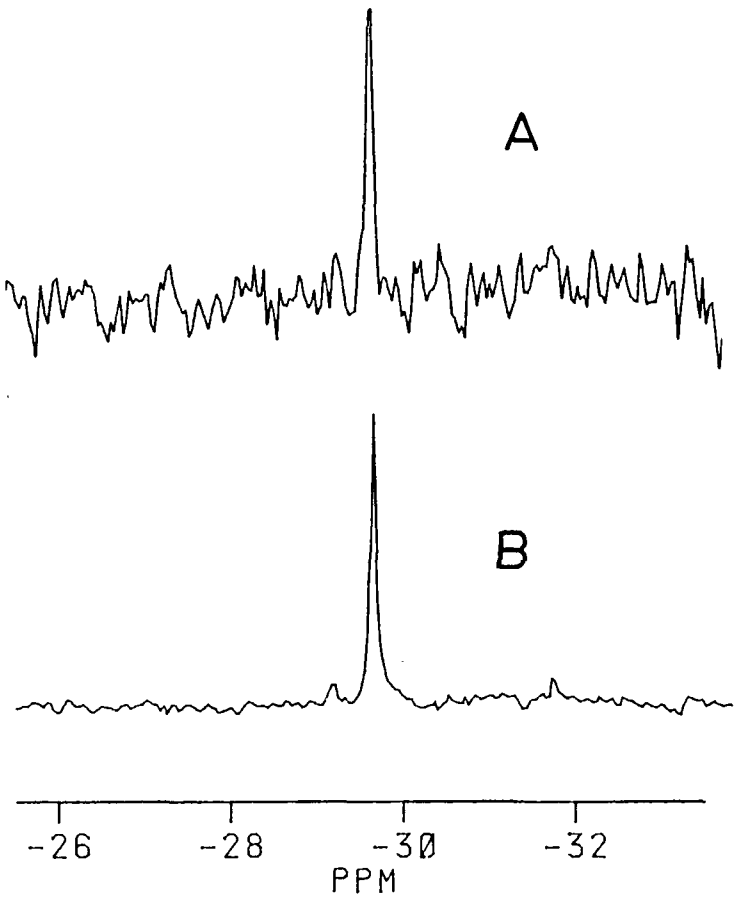


Fig. 10: ^{31}P-nmr spectrum of ethyl pinacolylphosphoric acid in the presence and absence of Cht. Measurements were performed in 0.1 M Tris chloride, pH 7.0. A) control; B) In the presence of 4 mM Cht. Negative signs indicate an upfield chemical shift relative to HMPA.

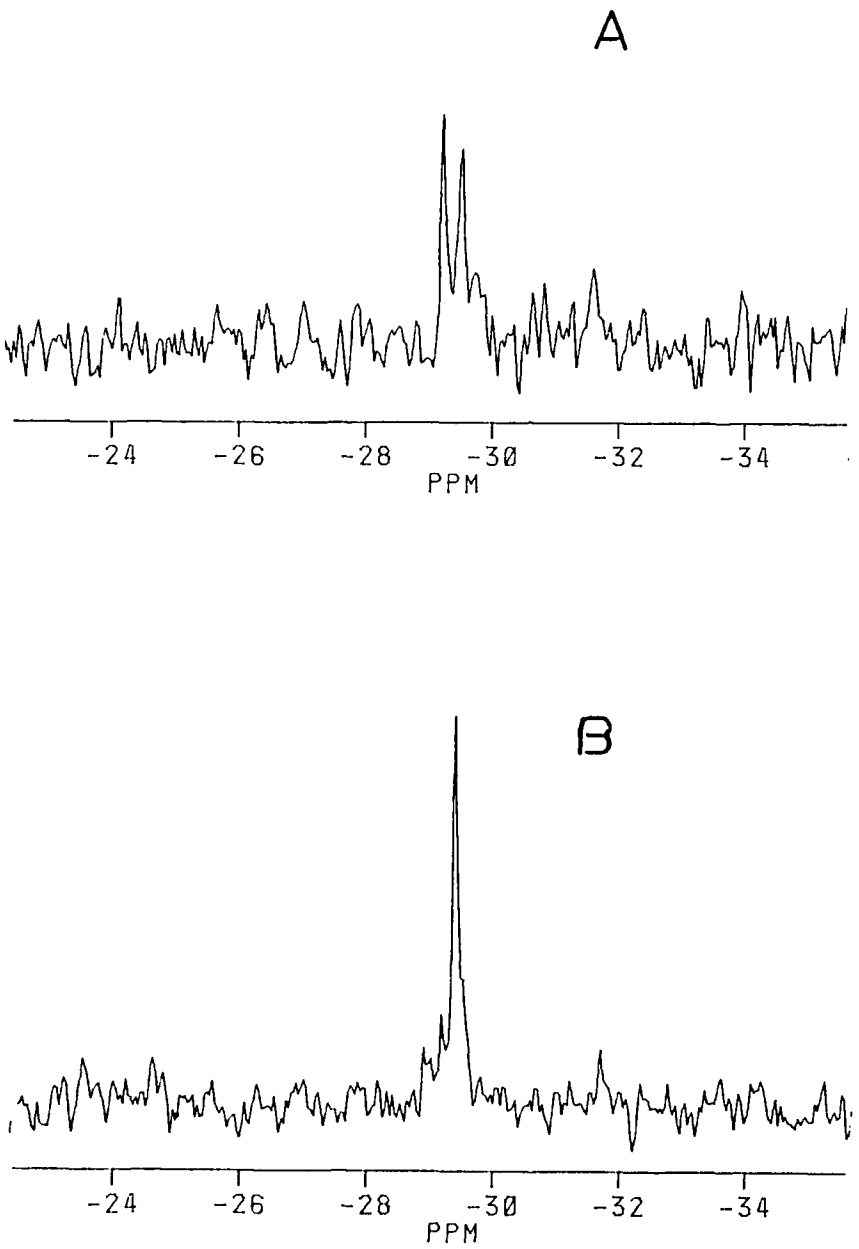


Fig. 11: Effect of prolonged heating on the ^{31}P-nmr spectrum of EPinP-Cht. The conjugate (1 mM) was incubated at 38°C for 120 h, in 0.1 M Tris chloride, pH 7.0. Spectra were recorded in the presence of 6 M Gu.HCl A) Heat-treated EPinP-Cht; B) Heat-treated EPinP-Cht + 2 mM ethyl pinacolylphosphoric acid. Dialysis of the mixture of EPinP-Cht and ethyl pinacolylphosphoric acid against 6 M Gu.HCl resulted in a ^{31}P-nmr spectrum identical to that shown in (A).

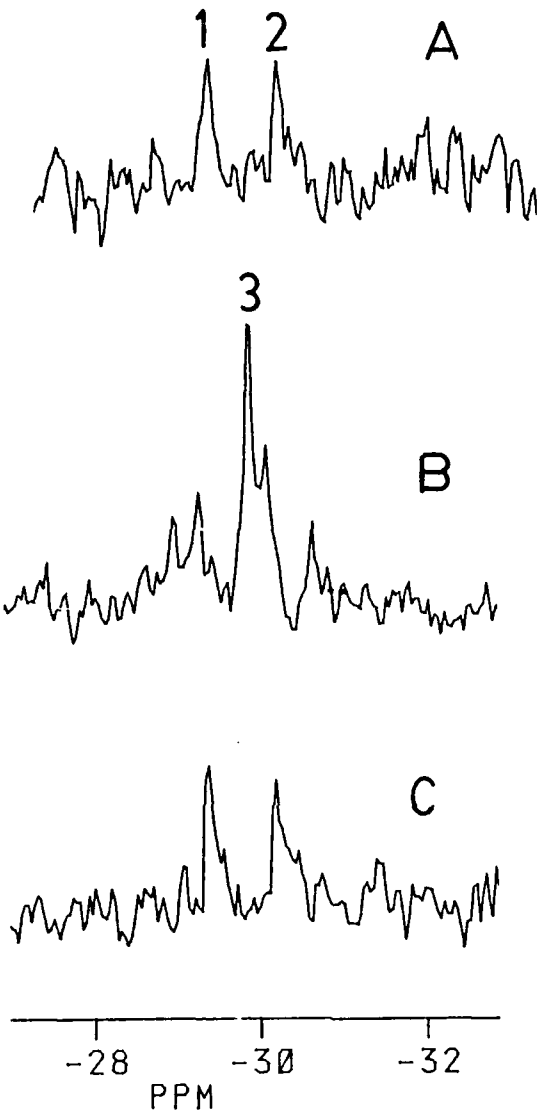


Fig. 12: Effect of prolonged heating on the ^{31}P-nmr spectrum of EIP-Cht. The conjugate (1 mM) was incubated at 38°C for 120 h in 0.1 M Tris chloride, pH 7.0. Spectra were recorded in the presence of 6 M Gu.HCl A) Heat-treated EIP-Cht; B) Heat-treated EIP-Cht + 2 mM ethyl isopropylphosphoric acid (peak 3); C) Sample (B) after dialysis against 6 M Gu.HCl Negative signs indicate an upfield chemical shift relative to HMPA.

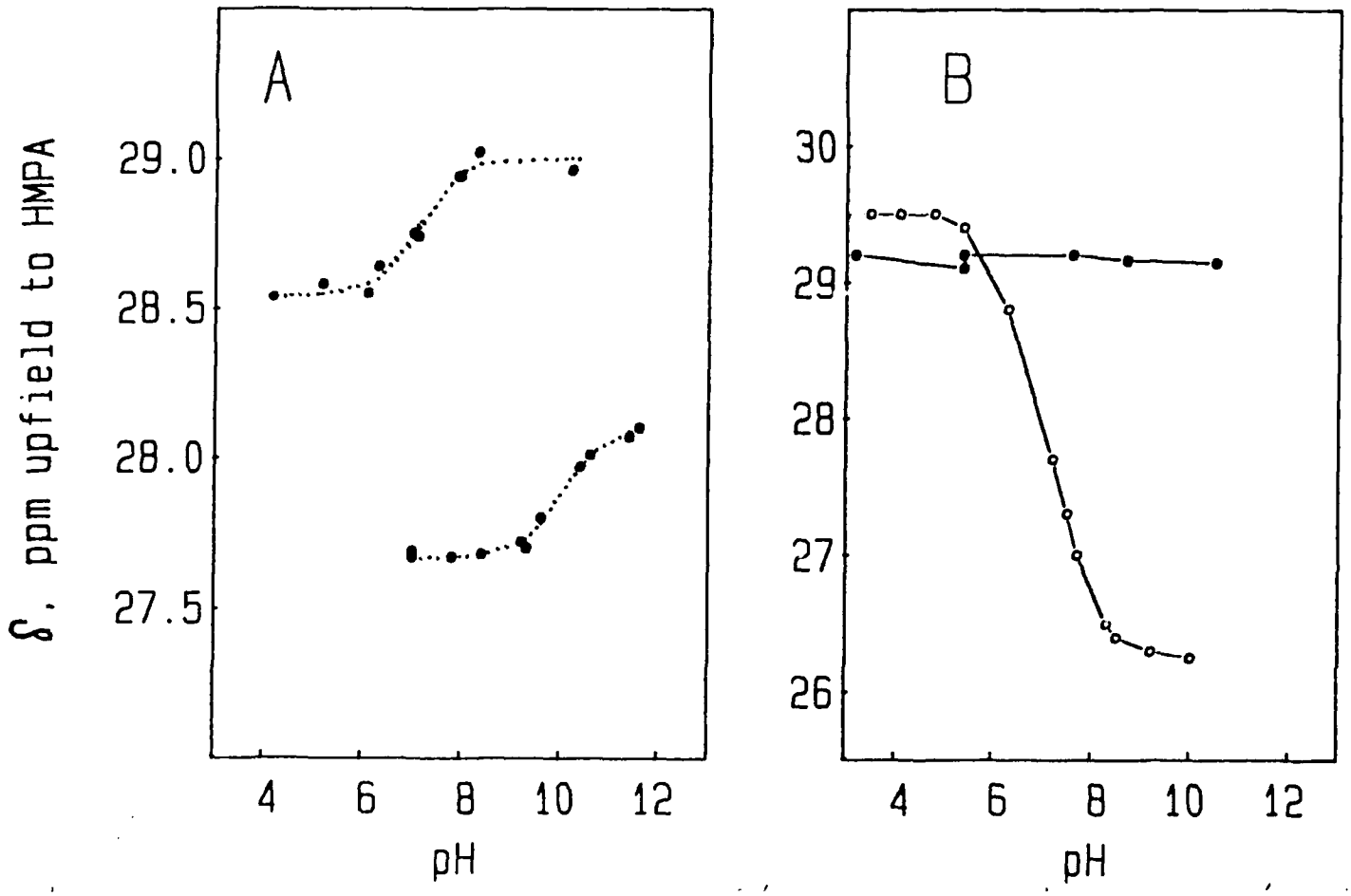


Fig. 13: pH-Dependence of ^{31}P-nmr chemical shifts of OP-Cht conjugates (A) and of phosphoric acid derivatives (B). The reported pH was not corrected for the isotope effect (25% D_2O). A) Upper curve, $(C_2H_5O)_2P(O)$-Cht (pK_a = 7.1); Lower curve, $C_2H_5OP(O)(O^-)$-Cht (pK_a = 10.3). B) ● - ● : $(C_2H_5O)_2P(O)(OH)$; O - O : $C_2H_5OP(O)(OH)_2$. The dotted lines in (A) were computer-fitted, assuming a single titratable residue.

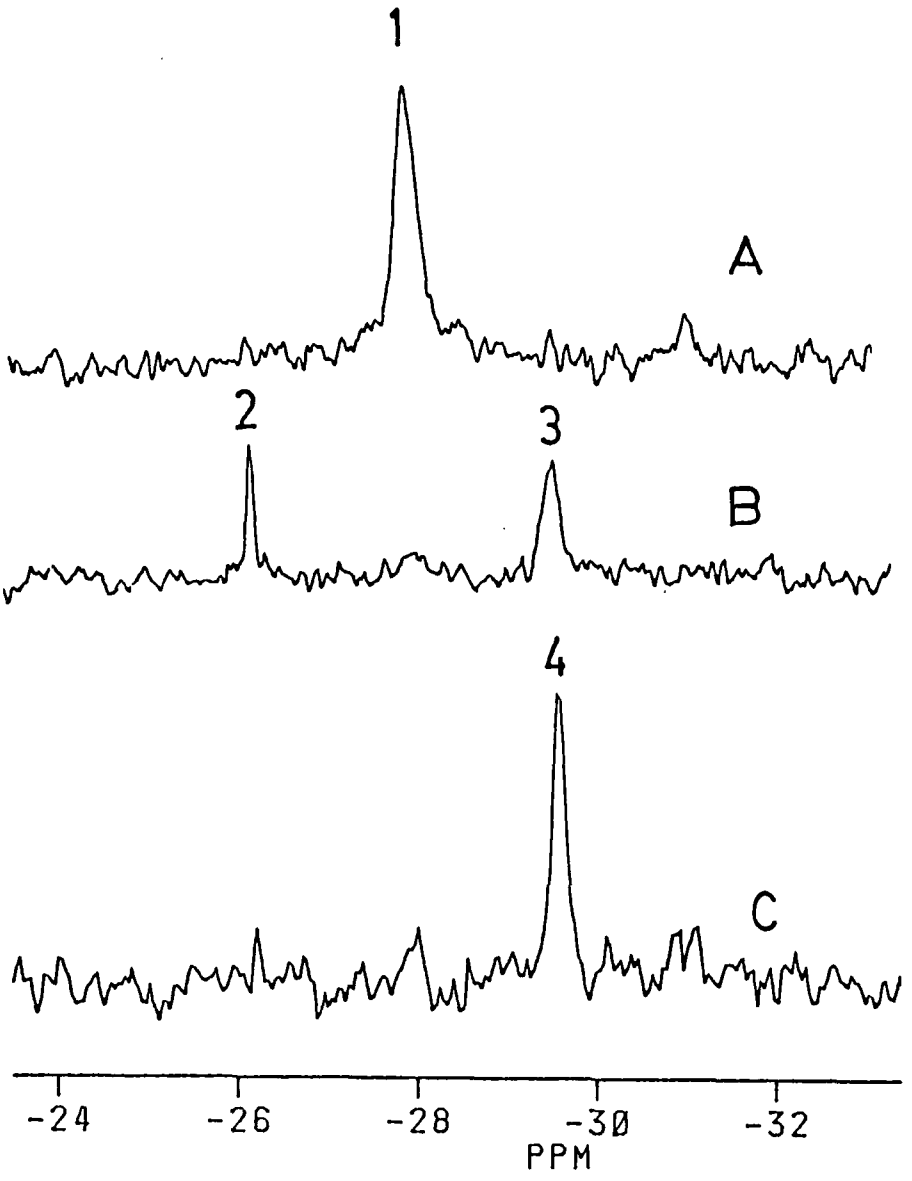


Fig. 14: Effect of 3-PAM at pH 10.5 on the ^{31}P-nmr spectrum of $C_2H_5OP(O)(O^-)$-Cht. The buffer employed was 0.1 M carbonate. A) 1 mM $C_2H_5OP(O)(O^-)$-Cht + 0.1 M 3-PAM after 48 h at 25°C; B) Same as (A), but after incubation at 38°C for 48 h; C) 1 mM $C_2H_5OP(O)(O^-)$-Cht after 48 h at 38°C in the absence of 3-PAM. Signals 1 and 2 correspond to undenatured $C_2H_5OP(O)(O^-)$-Cht and the diacid, $C_2H_5OP(O)(O^-)_2$, respectively. Signals 3 and 4 were identical with the ^{31}P-nmr chemical shift of $C_2H_5OP(O)(O^-)$-Cht denatured in 6 M Gu.HCl

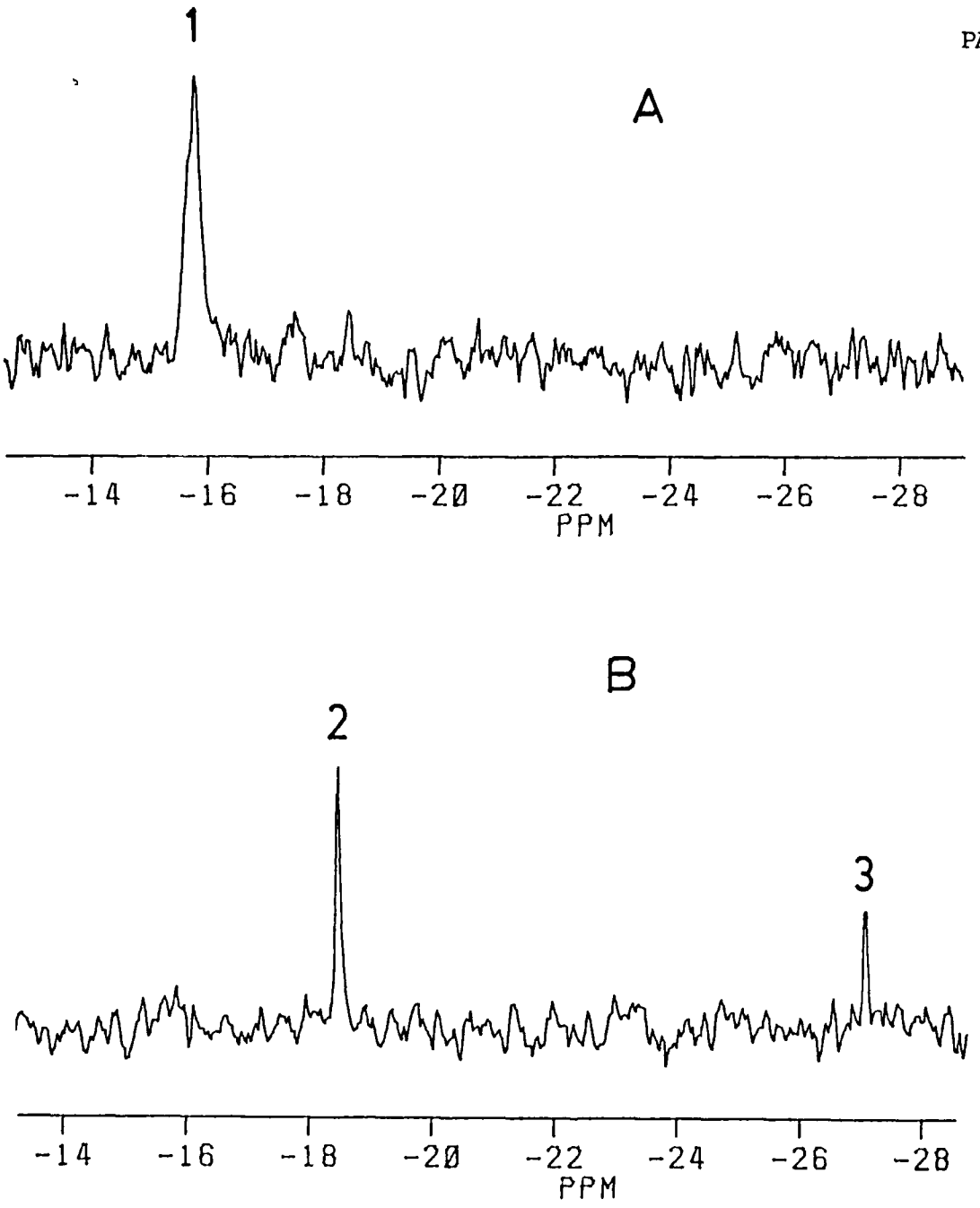


Fig. 15: Effects of high pH and 3-PAM on the ^{31}P-nmr spectrum of $(CH_3)_2NP(O)(OC_2H_5)$-Cht. Negative signs indicate an upfield chemical shift relative to HMPA. A) Spectrum recorded after 24 h in 0.1 M 3-PAM-0.1 M carbonate, pH 10.0; signal 1 corresponds to the native conjugate; B) A similar sample for which the spectrum was recorded after 24 h in 0.1 M carbonate, pH 11.4, in the absence of 3-PAM. Signals 2 and 3 were identified as the denatured form of $(CH_3)_2NP(O)(OC_2H_5)$-Cht and the acid, $(CH_3)_2NP(O)(OC_2H_5)(OH)$, respectively.

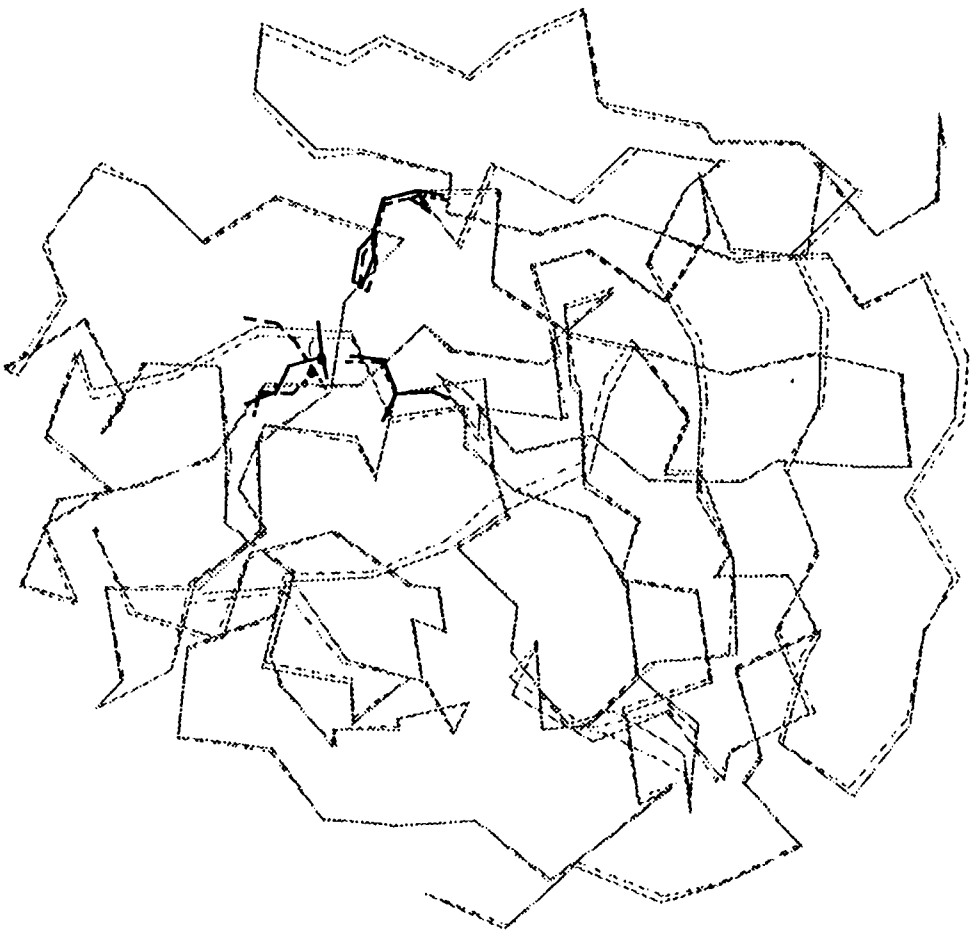


Fig. 16: Superposition of the polypeptide backbones of aged and non-aged OP-Cht conjugates: ------ , $(i\text{-}C_3H_7O)P(O)(O^-)$-Cht; - - -, $(C_2H_5O)_2P(O)$-Cht. The heavy lines, in each case, depict the positions of Ser-195, of His-57 and of the organophosphoryl residue.

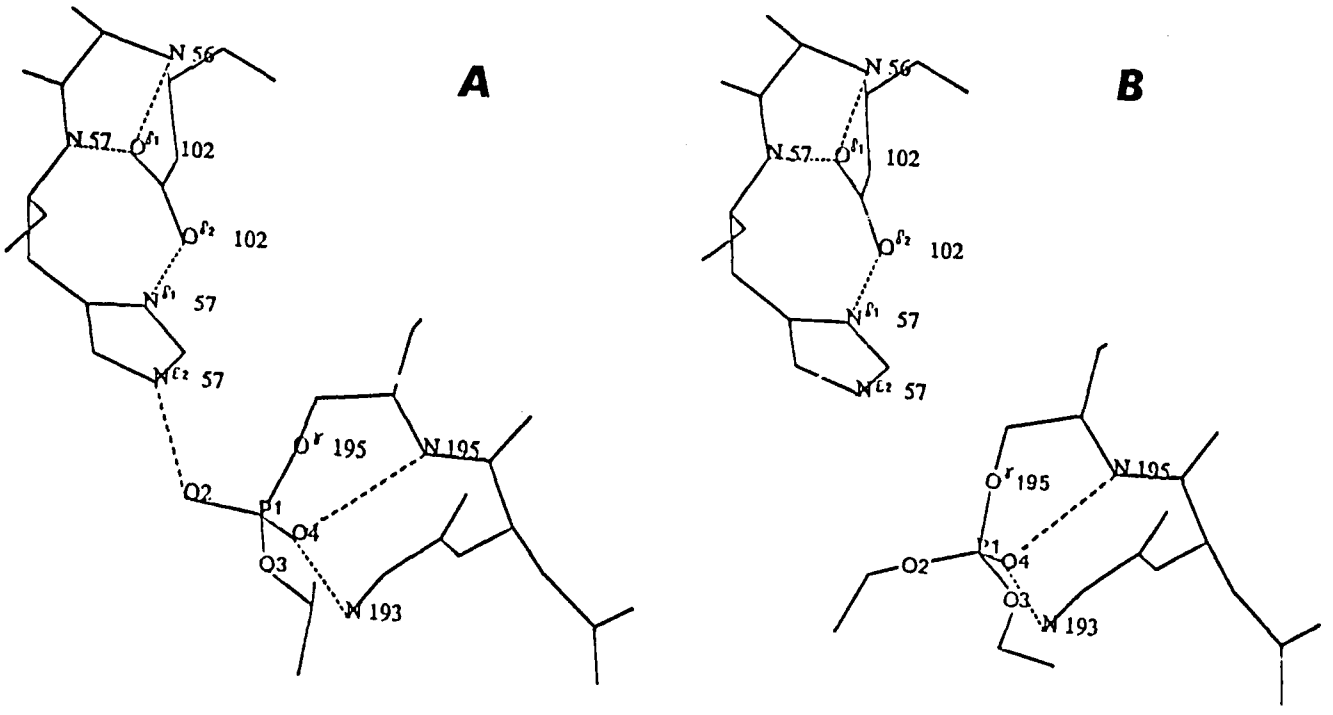


Fig. 17: Diagrammatic view of the active site regions of aged and non-aged OP-Cht conjugates as obtained from the refined X-ray data. A) The aged conjugate, $(i\text{-}C_3H_7O)P(O)(O^-)$-Cht; B) The non-aged conjugate, $(C_2H_5O)_2P(O)$-Cht. Residues Ala-56, His-57, Asp-102, Gly-193 and Ser-195 are displayed, as well as the bound organophosphoryl group. Selected atoms have been labelled so as to emphasize possible interactions (dashed lines) between atoms within the active sites of the phosphorylated enzymes.

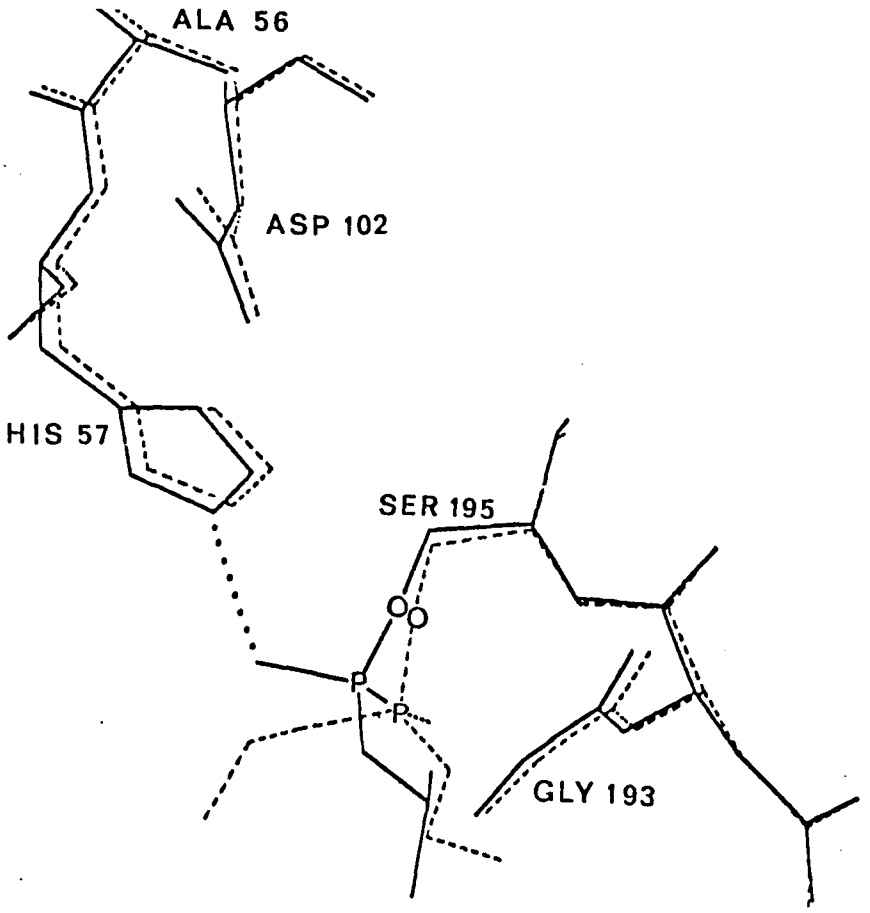


Fig. 18: Superposition of the active site regions of the aged and non-aged OP-Cht conjugates: ------, $(i\text{-}C_3H_7O)P(O)(O^-)$-Cht; - - -, $(C_2H_5O)_2P(O)$-Cht. The superposition shows that the protein backbone and sidechains within the active-site region do not change their positions markedly upon aging. The most prominent difference between the two conjugates is the movement of O2 on the phosphate group (for location see Fig. 17) towards His-57 $N^{\varepsilon 2}$ in the aged conjugate so as to form a hydrogen bond (.....).

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